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OBSERVATIONS ON THE INTOXICATION PRODUCED IN MICE AND RABBITS BY THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

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THE production of a toxin by *Shigella shigae* which paralyses rabbits and possesses the properties of a typical protein exotoxin has been established for the past half-century. Much of the work on it, as shown in the review of the extensive literature by Engley (1952), has been devoted to methods of preparation and to determining whether or not another toxin, the enterotoxin, was a separate entity. Recently, however, highly purified preparations of both toxins have been described. The enterotoxin, a typical complex of phospholipid, protein and polysaccharide, identical with the somatic antigen, was prepared by Davies, Morgan and Mosimann (1954) with a mouse LD_{50} of 80 μg. The neurotoxin obtained by van Heyningen and Gladstone (1953) was an electrophoretically homogeneous protein of molecular weight 82,000 and a mouse LD_{50} of 0·027 μg.

Less attention has been given to studying the site and mechanism of action of this potent exotoxin and its pathway of spread in the body. Shortly after the discovery of the neurotoxin Dopter (1905) described histological changes in the central nervous system of rabbits dying from the intoxication, the most important being chromatolysis of the anterior horn cells and interstitial haemorrhages. Although neuronal degeneration and haemorrhages were also observed by many early workers in the spinal axis of animals dying from botulism, Cowdry and Nicholson (1924) demonstrated that these changes were unrelated to the direct action of the toxin. Recent histological studies (Kishida, 1953; Bridgwater, Morgan, Rowson and Payling Wright, 1955) have confirmed, however, that Dopter's findings in shiga intoxication were due to the neurotoxin. The congestion of the intestines, frequently observed when less fractionated toxin preparations were used, is due to the associated enterotoxic somatic antigen (Olitsky and Kligler, 1920). Although such intestinal changes are non-specific, being producible by the comparable O antigens of other *Enterobacteriaceae*, the neurotoxin itself appears to be capable of producing some degree of bowel damage of a different type in certain animal species (Cavanagh, Howard and Whitby, unpublished).

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In view of the high order of toxicity of this neurotoxin, and the rarity of neurological symptoms in human shiga dysentery, it seemed of interest to apply to it some of the concepts used in determining the site of action and route of spread in the body of tetanus and botulinus toxins.

MATERIALS AND METHODS.

Toxin.

A freeze-dried sample of toxin, given by Dr. D. A. L. Davies of the Microbiological Research Department, Porton, was used. It was the lyophilised, dialysed autolysate from a culture of a rough strain of *Sh. shigae*. The LD_{50} following intraperitoneal inoculation of mice, determined by the method of Reed and Muench (1938), was 140 μg. Fresh solutions in physiological saline, in which the toxin readily dissolved, were made before each experiment.

Antitoxin.

The refined shiga dysentery antitoxin used contained 7000 units per ml. It was kindly supplied by Miss Mollie Barr of the Wellcome Research Laboratories, Beckenham. Dilutions were made in sterile saline before use.

Animals.

Adult lop-eared rabbits from the same stock, of either sex and weighing 3·0–4·5 kg. were used. The mice were an albino strain and weighed 18–22 g.

Anaesthesia.

Rabbits were lightly anaesthetised by giving " veterinary Nembutal " (25 mg. per kg.) intravenously, followed by open ether to obtain full surgical anaesthesia.

Operative techniques.

Mice.—Injections were made intraperitoneally, intramuscularly into the right thigh and intravenously into a tail vein. The volume of both toxin and antitoxin administered to mice by all routes was constantly 0·2 ml.

Rabbits.—Intravenous injections were made into a marginal ear vein. For injections into the medulla oblongata, the skin at the back of the neck was incised longitudinally, the muscles separated and the occipito-atlantoid membrane and meninges divided transversely. Escaping C.S.F. was absorbed with gauze and injections of 0·02 ml., made into the dorsum of the medulla with a tuberculin syringe fitted with a gauge 30 short-bevel needle. The sciatic nerve was exposed by incising the lateral aspect of the thigh and retracting the hamstring muscles. The nerve was mobilised and injected in the mid-femoral region with a tuberculin syringe fitted with a gauge 26 needle. The volume injected was 0·02–0·04 ml. The skin margins were approximated and sutured with silk thread. All operations were carried out aseptically and under full surgical anaesthesia.

RESULTS.

The Intoxication in Mice.

Injection of shiga neurotoxin into mice intravenously, intramuscularly or intraperitoneally produced a characteristic intoxication with a dosage range of 1–50 LD_{50}, irrespective of the inoculation route. An identical syndrome was produced by two other batches of toxin, obtained from different sources, and all symptoms were inhibited by the previous administration of antitoxin. The following description, based on observation of more than 300 mice, agrees in many features with that of Branham and Carlin (1948).

The first signs of intoxication to appear, after a lag period never less than 18 hr., were rapid breathing, ruffled fur, and a disinclination to move. The mice soon became excitable, walked unsteadily and developed a characteristic humping

of the back. Muscular weakness appeared in the hind limbs and tail, progressing to a flaccid paralysis. In the earlier stages of intoxication some mice had tetanic convulsions, which were frequently provoked by handling. Death was sometimes observed during a phase of rigid convulsion, but more usually the animal showed extreme flaccidity of the body musculature in the terminal phase.

It was early noticed that the neurotoxin proved more lethal when inoculated directly into the blood stream. Table I shows the number of deaths in groups

TABLE I.—*The Comparative Lethality of Shiga Neurotoxin Injected Intravenously, Intraperitoneally and Intramuscularly in Mice. Six Animals Inoculated in Each Group.*

Dose. (Calculated intraperitoneal LD_{50}.)	Number of deaths and mode of administration of toxin.		
	Intravenous.	Intraperitoneal.	Intramuscular.
1·0	6	5	4
0·85	6	1	0
0·7	5	1	0
0·55	3	0	0
0·425	4	0	0
0·3	2	0	0
0·15	0	0	0

of mice given similar graded doses of toxin intravenously, intraperitoneally and intramuscularly. Obviously the LD_{50} thus determined by the intravenous route will be significantly smaller than that calculated by the intraperitoneal method usually adopted. Again, the onset and progression of the intoxication were much more rapid and the median death time (Ipsen, 1941) shorter after intravenous toxin than after either intraperitoneal or intramuscular administration (Table II).

TABLE II.—*The Progression of the Intoxication Produced in Mice by the Administration of 5 LD_{50} Neurotoxin Intravenously, Intraperitoneally and Intramuscularly. Twelve Animals in Each Group.*

	Mode of administration of toxin.		
	Intravenous.	Intraperitoneal.	Intramuscular.
Condition at 24 hr.	4 dead 6 toxic 2 well	12 well	12 well
Condition at 48 hr.	9 dead 2 toxic 1 well	2 dead 8 toxic 2 well	1 dead 6 toxic 5 well
Median death time	38 hr.	60 hr.	64 hr.
Time for 12/12 deaths	72 ,,	96 ,,	92 ,,

The Effect of the Administration of Antitoxin During the Lag Period in Intoxicated Mice.

In view of the long period which elapses before signs of intoxication appear, the effect of intravenous injection of relatively large doses of antitoxin at varying intervals after the administration of toxin was investigated, to determine whether early tissue fixation of neurotoxin occurs. The difference in rapidity of toxic action, recorded above, necessitated giving the toxin by all of the three

TABLE III.—*The Effect of the Intravenous Administration of* 350 *Units Antitoxin, at Varying Intervals, on Mice Receiving* 5 LD_{50} *Neurotoxin Intravenously, Intraperitoneally and Intramuscularly. Six Animals Inoculated in Each Group.*

Period elapsed before giving intravenous antitoxin after toxin.	Number of deaths and mode of administration of toxin.		
	Intravenous.	Intraperitoneal.	Intramuscular.
½ hr.	4	0	0
1 ,,	4	0	0
2 ,,	6	0	0
6 ,,	5	1	1
24 ,,	—	5	4
18 hr. before toxin	0	0	0
No antitoxin given	6	6	6

same routes. Table III shows that the progress of fatal intoxication became irreversible very rapidly (within ½ hr. in some animals), when toxin was given by the intravenous route. If however the toxin had been given either intraperitoneally or intramuscularly it was still possible to afford good protection by giving antitoxin 6 hr. later. The inevitable fatal termination following intravenous toxin was in no way modified or postponed by increasing the already large dose of antitoxin previously used (Table IV).

The significance of these findings with mice in relation to the route of dissemination of neurotoxin in the body is considered in the discussion.

TABLE IV.—*The Effect of the Intravenous Administration of Varying Doses of Antitoxin, on Mice* 4 *hr. after Receiving* 5 LD_{50} *Neurotoxin Intravenously and Intraperitoneally. Six Animals Inoculated in Each Group.*

No. of units of antitoxin given 4 hr. after toxin.	Number of deaths and mode of administration of toxin.	
	Intraperitoneal.	Intravenous.
1400	—	6
700	1	6
350	1	5
100	3	6
10	2	6
1	5	—

The Intoxication in Rabbits.

The first signs to appear following the intravenous injection of 0·4–150 mouse LD_{50} toxin per kg. were an increased rate of respiration and failure to eat, following a lag period, with larger doses of toxin, of 10–12 hr. Muscular weakness first appeared in the fore limbs and, in lethal intoxications, proceeded to complete flaccid paralysis. At an early stage rabbits could not walk as their fore limbs splayed out and the involvement of the neck muscles prevented the animals from holding their heads erect. The abdominal musculature and hind limbs were progressively affected, leaving the rabbits paralysed with complete loss of muscle tone. The largest dose of toxin given intravenously killed a rabbit in 18 hr. No convulsive episodes comparable with those occurring in mice were observed,

but two rabbits with flaccid and paralysed fore limbs showed exaggerated hind limb reflexes. These disappeared rapidly as the paralysis progressed.

The Comparative Lethal Effect of Shiga Neurotoxin Injected Intravenously and into the Medulla Oblongata in Rabbits.

Tetanus neurotoxin exerts its most lethal effect by direct intramedullary inoculation, being approximately 1000 times more effective, assessed by death time, than when given intravenously (Wright, 1953). Rabbits given botulinus neurotoxin directly into the medulla however, survive longer than those receiving the same dose intravenously, the deaths being due to the ultimate absorption of toxin into the circulation (Davies, Morgan, Wright and Payling Wright, 1953). A comparable study has been made with shiga neurotoxin.

Four rabbits were injected intravenously with 0·75 mouse intraperitoneal LD_{50} toxin per kg., while another four animals received the same dose of toxin directly into the medulla oblongata. Table V shows that the period of survival

TABLE V.—*The Periods of Survival of Rabbits Injected with Shiga Neurotoxin Intravenously and into the Medulla Oblongata.*

Dose. Mouse i.p. LD_{50} per kg.	Period of survival.	
	Intravenous.	Intramedullary.
0·75	20 hr.	65 hr.
	38 ,,	100 ,,
	45 ,,	105 ,,
	55 ,,	Paralysis and recovery

was longer in the rabbits given intramedullary toxin. The fourth rabbit of the intramedullary group showed complete paralysis of the fore limbs at 80 hr. and weakness of the hind limbs at 95 hr. From this stage it improved progressively and showed full functional recovery at 192 hr. The symptomatology of the two groups in no way differed. If neurotoxin exerted its paralytic effect by a direct central action it should kill more rapidly by intramedullary inoculation than by intravenous. The result of injecting various doses of toxin intravenously emphasises that the period of survival may be much shorter than that recorded for the rabbits receiving intramedullary toxin (Table VI). The approximation of the death time for rabbits receiving 150 and 7·5 mouse i.p. LD_{50} per kg. suggests that 18 hr. probably represents the most rapid fatal intoxication producible in this species.

TABLE VI.—*The Periods of Survival of Rabbits Injected Intravenously with Varying Doses of Shiga Neurotoxin.*

Dose. Mouse i.p. LD_{50} per kg.	Period of survival.
150	18 hr.
7·5	20 ,,
	23 ,,
	24 ,,
0·4	67 ,,
	70 ,,
	81 ,,

The Effect of the Injection of Shiga Neurotoxin into the Sciatic Nerve of Rabbits.

Three rabbits (weight 3·3 kg.) were given 70 units of antitoxin intravenously and 3 hr. later 4 mouse i.p. LD_{50} toxin were injected into the left sciatic nerve trunk. None of the rabbits developed any neurological signs, nor indeed did any manifestation of intoxication appear. They were killed after 192 hr. This is contrary to the finding with tetanus where intrasciatic injection of toxin produces an ascending intoxication, in spite of the previous intravenous injection of large doses of antitoxin (Wright, Morgan and Payling Wright, 1951). Botulinus neurotoxin, however, resembles shiga toxin in producing no effect when injected similarly into a large nerve trunk (Davies *et al.*, 1953).

The Comparative Protection afforded by the Intrasciatic and Intravenous Inoculation of Antitoxin 18 hr. before giving Neurotoxin Intravenously.

Friedemann and his colleagues (Friedemann, Hollander and Tarlov, 1941; Friedemann and Traub, 1949) showed that tetanus antitoxin brought into contact with the cells of the central nervous system by injection into the cerebrospinal fluid afforded better protection against the toxin than if it had been given intravenously. Another method of bringing antitoxin into contact with the nerve cells is by intrasciatic injection, for Wright *et al.* (1951) showed that tetanus antitoxin would ascend the sciatic nerve to neutralise toxin given into the contralateral nerve. That molecules of comparable size can travel up the spinal cord to the brain stem is shown by the finding of the same workers that 16 hr. after the intrasciatic inoculation of radio-iodinated serum protein radioactivity was detectable in diminishing amount from the lumbar cord to the medulla oblongata. In the present experiment 18 hr. were allowed to elapse after the intrasciatic inoculations of shiga antitoxin before challenging with toxin.

The dose of antitoxin (140 units) was chosen so that by intravenous administration 18 hr. before giving 7·5 mouse i.p. LD_{50} per kg. it protected to the extent of allowing mild intoxication with recovery. Unprotected animals survived for 20–24 hr. only (*cf.* Table VI). Three rabbits received 140 units of antitoxin by injection into the left sciatic nerve and were challenged with the same dose of toxin intravenously 18 hr. later. They survived for 73, 73 and 107 hr., whereas of the 3 animals given the same dose of antitoxin intravenously, 2 developed only a slight fore limb weakness and the third a typical flaccid paralysis. All survived with no residual neurological signs. The protection afforded by intraneural antitoxin appears to be less than that following intravenous injection. The prolongation of the survival period following intrasciatic antitoxin, as compared with the unprotected control rabbits, may be attributed to some absorption of antitoxin into the circulation from the injection in the nerve trunk, for Wright *et al.* (1951) found a slow rise in radioactivity of the blood following the intrasciatic injection of radio-iodinated serum protein.

DISCUSSION.

No evidence that shiga neurotoxin, like tetanus toxin, can travel up nerve trunks to attack the central nervous system has been obtained in either mice or rabbits. Although Friedemann *et al.* (1941) found that up to eighty times more antitoxin is needed to neutralise tetanus toxin given intramuscularly than the

same dose injected intravenously, and d'Antona (1949) showed that an amount of tetanus antitoxin which would protect against intravenous toxin did not protect against intramuscular toxin, precisely the converse was found in the present experiments. In d'Antona's work previous division of the regional motor nerves allowed tetanus antitoxin to exert its full protective effect. The fact that antitoxin can protect mice six hours after giving shiga neurotoxin intramuscularly whereas a similar dose fails to protect mice half an hour after intravenous toxin is strong evidence against the possibility of a centripetal neural spread of toxin. The view is also supported by the total lack of signs of intoxication following the direct intrasciatic injection of toxin in rabbits. The absence of a demonstrable mechanism of nerve spread and the enhanced toxicity following the intravenous inoculation of mice strongly suggest that shiga neurotoxin is disseminated by the blood stream in animal intoxications.

Were shiga neurotoxin to exert its toxic effect by a direct action on central nerve cells, its lethality should be greatly enhanced by injection into the medulla oblongata, for, in rabbits, haemorrhage and neuronal destruction were observed by Olitsky and Kligler (1920) to be most prominent in the medulla and cervical cord. The period of survival of rabbits receiving intramedullary toxin was actually found to be considerably longer than that of animals given the same dose intravenously. It might be supposed alternatively, therefore, that the paralytic effect of the toxin is not produced in the central nervous system but sited peripherally, but this interpretation is incompatible with the histological disturbances in the spinal cord which have been frequently described. Furthermore, Bridgwater *et al.* (1955) could find no myographical evidence of disturbed function at the myoneural junction in intoxicated rabbits.

An interpretation which reconciles these findings is that the structural changes in the spinal axis are secondary to a damaging effect of the toxin on the neural vascular system. Direct support for this view is given by the observation of Bridgwater *et al.* that the damage in the spinal cord was far more extensive following the introduction of toxin into the vertebral artery than into the central nervous system itself. This is in conformity with the findings that neurotoxin on the circulatory side of the blood-brain barrier proves more potent than centrally-injected toxin, and that antitoxin given intravenously provides better protection than when injected intraneurally.

Why the neurotoxin should restrict its action to one selected group of blood vessels is obscure, but evidence will be presented later (Cavanagh, Howard and Whitby, unpublished) that this toxin is capable of producing vascular damage elsewhere in the body and that both the signs of intoxication and the distribution of lesions found vary in different animal species.

SUMMARY.

The lethality of shiga neurotoxin for mice is greater, and the progress of intoxication accelerated, when injected intravenously than either intramuscularly or intraperitoneally. The intoxication becomes irreversible by a subsequent intravenous injection of antitoxin if the latter is delayed for as little as half an hour, although toxic signs do not appear for 18 hours. On the other hand, after injecting toxin either intramuscularly or intraperitoneally good protection is obtained with antitoxin given up to 6 hours later.

Rabbits survive longer when the neurotoxin is injected into the medulla oblongata than when the same dose is given intravenously. Direct injection of toxin into the sciatic nerve of rabbits is without effect. The prophylactic action of antitoxin is greater when given intravenously than when injected into the sciatic nerve.

It is considered that the pathogenesis of shiga intoxication depends on dissemination of the neurotoxin by the circulation and that neuronal destruction cannot be attributed to a direct action of toxin on the nerve cells. The primary site of attack is discussed.

I am grateful to Dr. D. A. L. Davies and Miss Mollie Barr for gifts of shiga neurotoxin and antitoxin, and to Professor G. Payling Wright and Dr. W. E. van Heyningen for valuable discussions during the course of this work.

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THE NEUROTOXIN OF *SHIGELLA SHIGAE*.

MORPHOLOGICAL AND FUNCTIONAL LESIONS PRODUCED IN THE CENTRAL NERVOUS SYSTEM OF RABBITS.

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THE earliest descriptions of neurological disturbances caused by the intravenous injection of toxic filtrates of cultures of *Shigella shigae* were given by Vaillard and Dopter (1903). Two years later Dopter (1905) described in detail the morphological changes in the spinal cord developing in the course of this intoxication. The multiplicity of the toxic constituents of these and similar *Sh. shigae* filtrates, however, much complicated the efforts of several later investigators to analyse their individual activities and separate their distinctive enterotoxic and neurotoxic fractions (see Engley, 1952). Recently, van Heyningen and Gladstone (1953) succeeded in effecting a much more complete separation of the neurotoxin than had been accomplished previously, and obtained a material which in its lethality for rabbits vied with botulinum and tetanus toxins. It was with this preparation, for a supply of which we are greatly indebted to these authors, that the following observations on the mechanism of action of this dysentery neurotoxin were made.

METHODS.

Toxin.

The preparation of toxin used was part of the material whose extraction and purification from the culture of a rough strain of *Sh. shigae* has been fully described by van Heyningen and Gladstone (1953). Its very high toxicity for rabbits can be seen from the Table; from these findings its calculated LD_{50} for rabbits on intravenous injection was 0·023 μg. In all references to dosages below, irrespective of route of inoculation, this value for the LD_{50} is the unit used. This estimate is ten times higher than that given by van Heyningen and Gladstone—a difference perhaps attributable to a decline in potency on storage for over two years in glycerol-saline solution. Like several previous authors (see Howard, 1955), we have found this toxin to be much less lethal for mice and guinea-pigs than for rabbits.

TABLE—*Lethality of* Sh. shigae *Neurotoxin for Rabbits on Intravenous Injection.*

Dose. (μg.).	Number of rabbits. Injected.	Dead within 96 hr.
0·0115	4	0
0·0230	18	8
0·0345	3	3
0·0460	6	6

Antitoxin.

The preparation used was Wellcome anti-dysentery serum (Shiga) unconcentrated, 2000 units/ml.

Animals.

Except for a few experiments in which guinea-pigs and mice were used, the observations were made on albino rabbits of 2–3 kg. weight.

Operative techniques.

Intravenous inoculations were made into the marginal vein of the ear; to ensure accuracy of injection, the rabbits were lightly anaesthetised with "veterinary Nembutal". Intra-arterial inoculations were made into the right vertebral artery near to its origin from the subclavian artery. The operation was carried out with aseptic care under full Nembutal and ether anaesthesia. When the artery had been identified and exposed adequately, it was ligated close to its source and the inoculation made into a distal part of the vessel. To exclude the possibility that any lesions found subsequently in the cervical spinal cord might have been due to local ischaemia, control inoculations were made in an exactly similar manner with fully neutralised toxin-antitoxin mixtures. Intramedullary injections into the medulla oblongata were made by the method described by Davies, Morgan, Wright and Payling Wright (1953). Intraneural inoculations into the sciatic nerve were made by the method described by Wright, Morgan and Payling Wright (1951).

In some rabbits exposed to general or local intoxication, mechanograms were obtained from the flexor carpi ulnaris muscle after detaching its tendon from its insertion in the carpus and connecting it to a torsion-wire myograph which recorded changes in tension by a lever on a smoked drum. These muscles were excited indirectly by a square-wave pulse of 1 m.sec. duration and of twice maximal intensity applied at intervals of 2 sec. to the ulnar nerve about the mid-humeral level through platinum wire electrodes.

Histological methods.

After fixation in formol-alcohol, paraffin-embedded sections were stained by haematoxylin and eosin, by cresyl violet for Nissl substance, by Glees and Marsland's modification of Bielschowsky's method for axons. Peripheral nerves were also examined by Swank and Davenport's modified Marchi's method for Wallerian degeneration. Pieces of brain and spinal cord were also fixed in formol saline, cut in sections 150 μ thick on the CO_2 freezing microtome and stained by Pickworth's benzidine method for evidence of haemorrhages.

RESULTS.

Injections of Toxin Intravenously.

General manifestations.

There was considerable variability in the severity of the intoxication induced by intravenous inoculations of doses of toxin that were near to the LD_{50} value: some of the animals seemed to be little affected, while others injected at the same

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EXPLANATION OF PLATES.

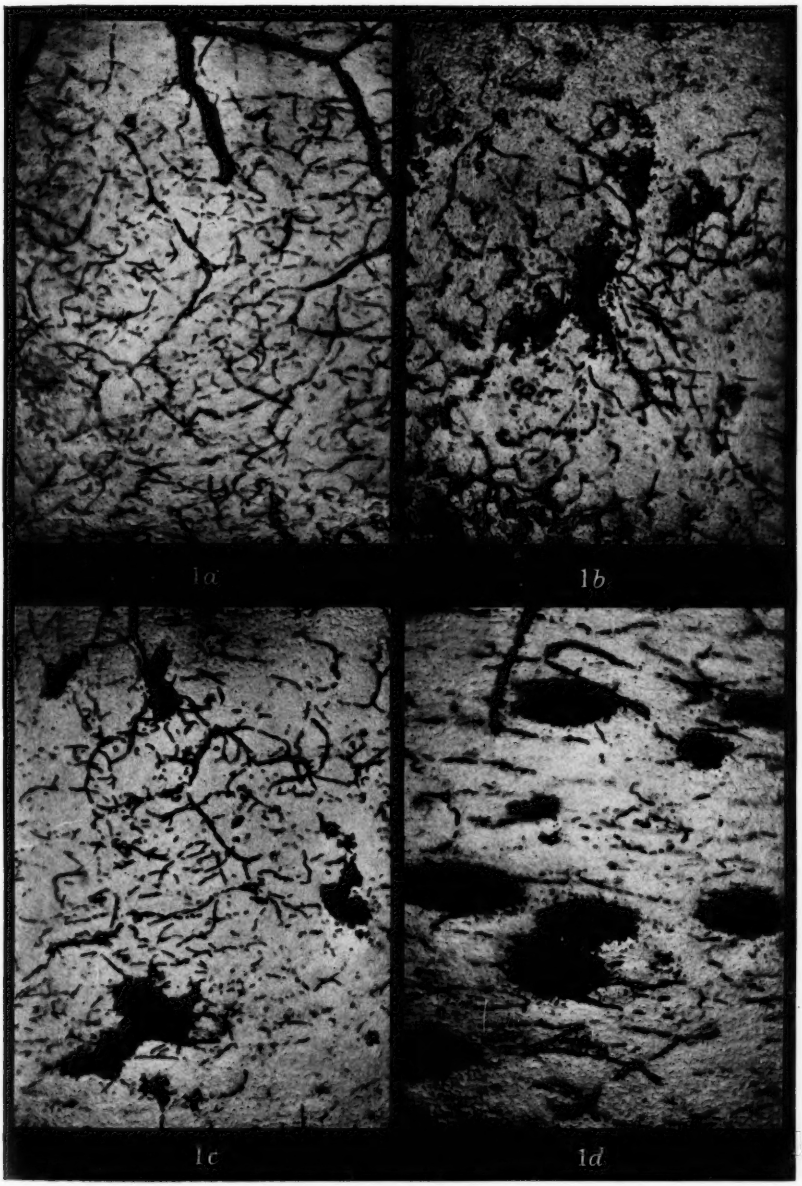
FIG. 1.—Section from cervical spinal cords of rabbits at varying stages of intoxication: (*a*) control; (*b*) 24 hr.; (*c*) 48 hr.; (*d*) 72 hr. Stained by Pickworth's benzidine method. × 50.

FIG. 2.—Anterior horn cells of rabbits at different stages of intoxication: (*a*) control; (*b*) 72 hr.; (*c* and *d*) 120 hr. Stained with cresyl violet. × 850.

FIG. 3.—Small focal haemorrhage near an apparently normal anterior horn cell in an intoxicated cervical spinal cord. Stained with cresyl violet. × 850.

FIG. 4.—Longitudinal sections from rabbit's cervical spinal cords: (*a*) control; (*b*) after 120 hr. intoxication. Stained by Glees and Marsland's method. × 50.

FIG. 5.—Longitudinal section from cervical spinal cord of rabbit 120 hr. after injection of toxin into right vertebral artery, showing destruction of grey matter on that side. Stained with cresyl violet. × 10.



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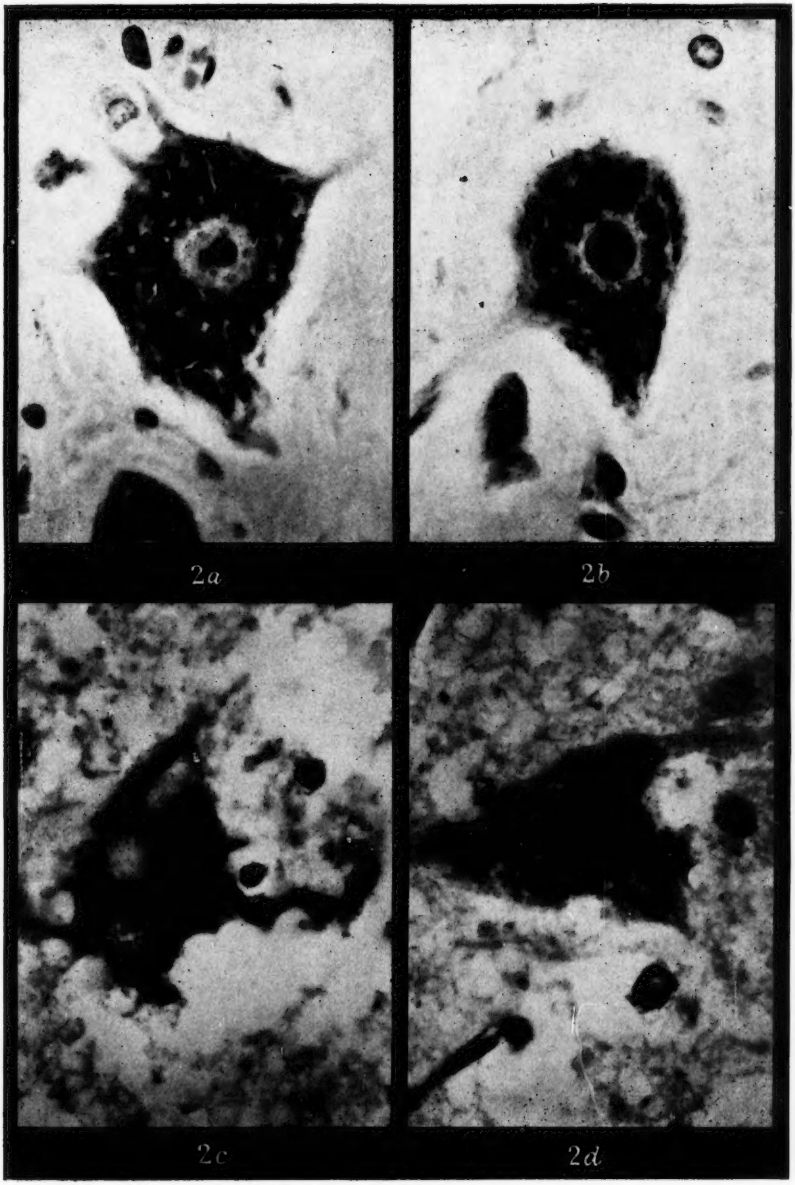

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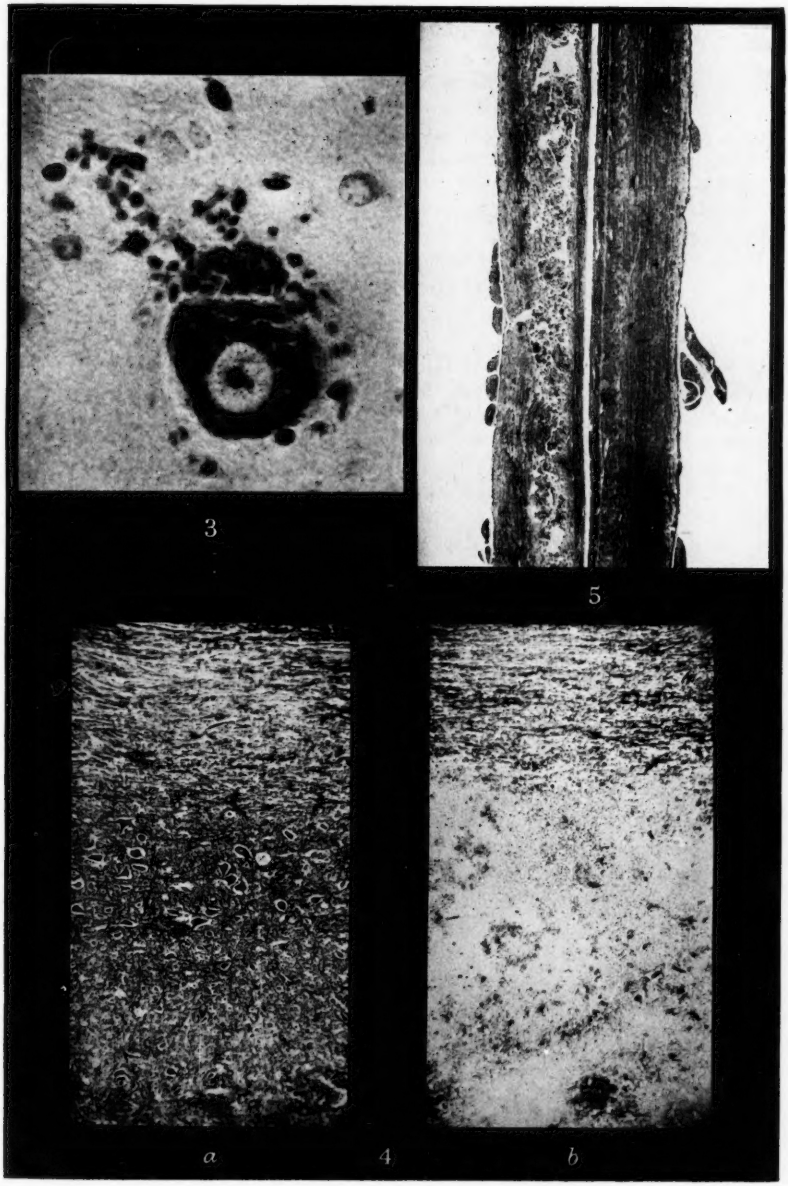

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a 4 *b*

Bridgwater, Morgan, Rowson and Payling Wright.

time were severely and often fatally intoxicated. With larger doses, the toxin was uniformly lethal. Even when the intoxication eventually proved fatal, however, the rabbit showed no overt evidence of injury for the first two days. About that time, the first signs of paresis appeared, generally in the fore limb, and became accentuated after mild exercise. During the ensuing 24 to 48 hr., the degree of paralysis usually increased and often extended to the hind limbs. By the fourth or fifth day, such rabbits were prostrated and hypothermic ; often they had also developed marked head retraction. With the largest doses of toxin used (2 LD_{50}) some developed terminal diarrhoea. Even without special care and attention, however, some of the animals prostrated at the height of the intoxication made surprisingly rapid recoveries, so that after a further 48 hr. little or no sign of their former paresis remained.

Post-mortem appearances.

Rabbits which had received 2 LD_{50} and had died or were killed at the height of the intoxication showed gross macroscopic changes in the spinal cord. Especially in the cervical, but sometimes also in the lumbar enlargement, the cord was swollen and on section extensive areas of softening and numerous petechial haemorrhages were visible in its grey matter. The white matter, even in the vicinity of the most severely disrupted grey matter, appeared to be little affected and sometimes formed the wall of a tube-like structure whose contents were almost pultaceous. Corroborative evidence for the integrity of much of the white matter round such lesions is provided by the occasional absence of hind limb paralysis in animals whose cervical enlargement had been severely damaged.

In some of the rabbits which had received large doses of toxin, the mucosa of the caecum was oedematous and speckled with haemorrhages—some of them petechial, others confluent. In none of the other major organs were haemorrhagic or other lesions observed.

Histological changes.

In rabbits which had received 2 LD_{50} of the toxin, and were killed 24 hr. later without their having presented any external signs of intoxication, lesions could be recognised in the central nervous system. Small haemorrhages, some apparently arising from capillaries and others from small veins, were present in the cervical enlargement of the spinal cord and less often in the cerebellum. These extravasations of blood, which were clearly displayed in thick frozen sections stained by Pickworth's benzidine method (Fig. 1), were much more numerous in the grey than in the white matter—a difference probably attributable to the much greater vascularity of the former (see Krogh, 1945). At this early stage of the intoxication, the large perikaryons of the cervical spinal motor nuclei showed no morphological sign of injury. When stained with cresyl violet, these cell bodies showed no chromatolysis, even when they lay close to the sites of petechial haemorrhages (Fig. 3).

When rabbits were killed after 48 hr., even though no signs of any functional deterioration had yet appeared, the vascular disturbances in the spinal cord were more pronounced. Petechial haemorrhages, especially in the grey matter, were both larger and more numerous (Fig. 1) ; again they were more marked in the cervical than in the lumbar enlargement. At this stage a small proportion of the large motor perikaryons in the anterior horns showed signs of injury ; the

Nissl material stained less intensely, and some neuronal nuclei showed early pyknosis. The great majority of these anterior horn cells, however, seemed to be unaffected by the concomitant changes in the blood vessels even when they were close to sites of petechial haemorrhage.

After 72 hr., when the rabbit presented grave pareses of its fore limbs and often also of its hind limbs, the haemorrhages into the spinal cord were conspicuous (Fig. 1), and had created small foci of structural disruption. Many of the large motor neurones now exhibited clear evidence of injury ; some showed marked chromatolysis, occasionally with vacuolisation of their cytoplasm, while in others the nuclei were pyknotic and separated from the remains of the Nissl substance by a clear relatively unstained halo (Fig. 2). Even at this stage, however, most of the neuroglial cells showed few signs of damage, though occasionally small aggregations of microglial cells could be seen.

When profoundly intoxicated rabbits were killed after 120 hr. the evidence of destruction was widespread and involved cells of all types. The severity of the injury is shown in Fig. 4, in which sections, stained by a silver impregnation technique for the display of cells and axons, taken from the cervical enlargement of the spinal cord of a normal rabbit and one that had been intoxicated for 120 hr., are contrasted. In the latter, the region of the grey matter was occupied only by cellular and neuropil débris, the neurones, neuroglia and blood vessels all having necrosed.

Several rabbits which had received only 1 LD_{50} recovered full mobility after temporary paralysis and were killed 12–14 days after they had been inoculated. In these animals there was little evidence of injury to either the grey or white matter of the spinal cord, although small areas of microglial aggregations were occasionally observed in the former. Moreover, marked perivascular cuffing was present round small arteries. The great majority of the motor perikaryons appeared normal histologically, though some must have been lost, for evidence of Wallerian degeneration was present in a small proportion of fibres in the main motor nerve trunks of both the upper and the lower limbs.

Injections of Toxin into the Vertebral Artery.

Three rabbits were given 2 LD_{50} of the toxin in 0·25 ml. saline directly into the right vertebral artery, and two further rabbits, which served as controls for any purely vascular disturbance caused by the procedure, were injected by the same route with the same dose of toxin which had been previously fully neutralised with specific antitoxin. One of these control animals developed slight weakness in the right fore limb 24 hr. later, but had fully recovered by the following day. Both control animals were observed daily until they were killed 14 days later.

The rabbits which had received unneutralised toxin remained apparently normal for the succeeding two days when they began to develop weakness of their fore limbs, especially that on the right side. The state of intoxication steadily progressed to one of prostration, the hind limbs became weaker, head retraction developed and respiration became embarrassed. Post mortem, the severity of the injury inflected on the spinal cord became apparent when the cervical enlargement was exposed by a longitudinal section. In Fig. 5, taken from such a spinal cord, the remarkably sharply demarcated unilateral distribution of the necrosis is clearly seen. This restriction of the injury to the side on which the

injection was made accords well with the observations of McDonald and Potter (1951) who found that dye introduced into the vertebral artery stained the ipsilateral half of the spinal cord from the first thoracic segment to the junction of the medulla oblongata and the first cervical segment. On histological examination, the whole of the grey matter on this side of the cord was found to have undergone necrosis, only cellular and neuropil débris being visible.

Injections of Toxin into the Medulla Oblongata.

In five rabbits, the toxin, varying in amount from 0·4 to 1 LD_{50}, was injected directly into the medulla oblongata close to the obex. Four of these animals developed signs of brain-stem lesions, ataxia, bradycardia and epileptiform attacks after 48 hr. but none showed any paralysis in either the fore or hind limbs. One rabbit which had received 0·5 LD_{50} remained apparently normal throughout, as did two animals which were given 1 LD_{50} of toxin neutralised with antitoxin.

At post mortem there were localised areas of necrosis 2–3 mm. in diameter around the site of injection in all rabbits given toxin only. The minor variations in location inevitable with such injections, and their differing relations to the brain stem nuclei, probably accounted for the varying severity of the accompanying functional disturbances. The boundaries of these areas were fairly sharply demarcated, and the narrow zone that separated the necrosed from the remaining medullary structures was often marked by small haemorrhages. Histologically all the cells in the affected area, irrespective of type, had been destroyed. The focal character of the lesion was emphasised by the apparently normal morphology of many neurones close to the margin of the necrosed areas.

In the two rabbits given the toxin-antitoxin mixture, only small haemorrhages and leucocyte aggregations, distinctive of minor trauma, were visible along the needle track.

Injection of Toxin into Nerve Trunks.

In 2 rabbits, toxin in doses of 1 and 2 LD_{50} was injected directly into the left sciatic nerve; the animals were observed for the following eight days, and no sign of functional disturbance appeared in either. In 2 other rabbits, an injection of 2 LD_{50} of toxin was made into the right brachial nerve. One of these animals developed some weakness of the fore limbs on the 4th day: the other remained normal until both were killed on the 6th day. Histological examination of the cervical enlargement of the spinal cord in the former showed some small petechial haemorrhages and microglial aggregations.

The Effect of Toxin on Peripheral Neuromuscular Conduction.

The possibility that in addition to its central neurotoxic action, the toxin might have some injurious effect on peripheral somatic nerve endings in muscle was examined, firstly, in rabbits which were in a state of prostration as the result of an intravenous injection, and secondly, in rabbits in which the injection had been made locally into the belly of the flexor carpi ulnaris muscle.

Three rabbits which had received 1–1·5 LD_{50} of the toxin intravenously showed marked paralysis of the fore limbs three days later. When the contractile power of the flexor carpi ulnaris muscle in these animals was tested by the electrical stimulation of the ulnar nerve, they contracted as strongly as the same muscle in a normal rabbit. This normal contractility in shiga neurotoxin intoxication

contrasted with the marked loss of strength in this muscle in a rabbit given a sublethal intravenous injection of Type A botulinum toxin.

In two rabbits a solution of shiga neurotoxin containing 30 LD_{50} in 0·2 ml. saline was injected at four spaced sites in the flexor carpi ulnaris muscle under light Nembutal anaesthesia. In one of these rabbits, the contractile strength of this muscle and that of its opposite uninjected control muscle was tested 6 hr. later, and in the other, the same comparison was made after 42 hr., by which time definite signs of general intoxication had appeared. In neither rabbit did indirect stimulation through its motor nerve disclose any weakness in the inoculated muscle. To test the effectiveness of this procedure for producing the local intoxication of a muscle, 10,000 mouse LD_{50} Type A botulinum toxin in the same volume of saline was injected in the same way. Six hours later, by which time local paralysis seemed complete, the stimulation of the ulnar nerve no longer brought about a contraction.

DISCUSSION.

The occurrence of paralysis in the limbs of rabbits after the injection of shiga dysentery bacilli and their culture filtrates has long been recognised and the likelihood of its dependence on lesions in the spinal cord has been generally accepted since the studies of Vaillard and Dopter (1903). From time to time, however, the possibility has been canvassed that these effects might be fortified by some additional intoxication at the peripheral endings of the somatic motor nerves of a kind akin to that found in botulism. This latter suggestion receives no support from the present investigations: the effects seem to be accounted for fully by the highly destructive lesions produced by the toxin in the grey matter of the spinal cord.

The literature on the intoxications caused by products of the shiga dysentery bacillus has been much complicated by the multiplicity of the toxic constituents in its culture filtrates (see Engley, 1952). Although, from the descriptions of the lesions they observed, it is clear that many of the earlier investigators were employing preparations capable of gravely injuring the nervous system, it is only recently that the purification and concentration of this neurotoxin effected by van Heyningen and Gladstone (1953) has enabled the more specific actions of this agent to be distinguished from those of other toxic products of this organism.

Although in the highly susceptible rabbit, the brunt of the intoxication falls on the spinal cord, and notably on its two enlargements, in less vulnerable species the main nervous lesions may develop elsewhere. Kishida (1953), while studying the effects of intravenous injections of living and dead cultures of *Sh. shigae*, noted that although in rabbits the lesions were mainly confined to the spinal cord enlargements, in monkeys they were less common in the spinal cord and much more numerous in the cerebral cortex, cerebellum and basal ganglia. It would seem, therefore, that in different species, some local factor must determine the sites at which the toxin will have its maximal effect.

Although in rabbits the lesions are almost confined to the central nervous system, it is not easy to decide which type of cell in the cerebrospinal axis is the first to suffer injury. From the above described sequence of events in the intoxication, however, there seem to be grounds for believing that this neurotoxin differs from those of botulism and tetanus in that its primary action is not on nerve cells but rather on the endothelium of the smaller blood vessels of the

central nervous system. In short, this toxin appears to be a secondary rather than a primary neurotoxin—a possibility also envisaged by Doerr and Seidenberg (1937) in their attempt to use it to effect the localisation of certain viruses in the central nervous system. Throughout the early stages of the intoxication, long before any signs of the characteristic paralysis have yet appeared, the neuronal changes appear to lag behind the slowly but steadily progressive vascular lesions, so that it becomes difficult to draw any other inference than that the former are the sequel to the latter. Eventually, if time is permitted for the action of a lethal dose of toxin, all the elements of the grey matter are involved in a common ruin.

If this interpretation of the sequence of events is correct, two questions emerge to neither of which has any satisfactory answer yet been suggested. Why is the interval between the injection of toxin and the onset of paralysis two days or more, and why in rabbits are the vascular lesions restricted to the grey matter of the spinal cord enlargements? In regard to the former, it may be noted that a delay in the appearance of capillary vascular lesions is not unique for the shiga neurotoxin, for the erythematous reactions in the Schick and Dick tests only become recognisable many hours after the injection of the respective toxins. No explanation for the slow action of shiga neurotoxin in terms of delay in fixation to the target organ appears likely, however, for Howard (1955) has observed, and our findings have been similar, that a subsequent intravenous injection of antitoxin is protective only if given within 30 minutes of the toxin. Why this neurotoxin should prove so selectively injurious to the smaller blood vessels of the central nervous system is wholly obscure. That it is not solely related to the physiological peculiarity of this endothelium which determines the haematoencephalic barrier seems apparent from the restricted distribution of the haemorrhages and their differing locations in the neuraxes of different species.

SUMMARY.

The intravenous injection of a lethal dose of purified shiga neurotoxin in rabbits is followed by the appearance of progressively increasing focal haemorrhages in the grey matter of the spinal cord enlargements. These extravasations of blood first arise long before the animals present any neurological disability, and precede by many hours the appearance of injury to the nerve cells of the spinal motor nuclei. While selectively destructive for the central nervous system in rabbits and other animals, this agent, in distinction to botulinum or tetanus toxin, may be regarded as a secondary neurotoxin. There is no evidence that it has any paralytic action on peripheral motor nerve endings.

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THE SULPHUR REQUIREMENT OF RAT CONNECTIVE TISSUE CELLS.

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In a previous publication (Harris, 1955) a technique was described for measuring accurately the rate of multiplication of rat connective tissue cells. It was found that the growth rate of these cells in diluted serum was at no time logarithmic, and growth ceased after 70 to 80 hours despite repeated changes of medium. In the present study the factors limiting the multiplication of these cells were more closely investigated. It was shown that a limiting factor for growth in serum was the availability of certain sulphur compounds.

EXPERIMENTAL.

The connective tissue cells were obtained from explants of the heart muscle of 5-day-old albino rats. The cells were cultivated in special chambers which permitted serial counts to be made directly by phase contrast microscopy. The details of the experimental procedure have been given elsewhere (Harris, 1955).

RESULTS.

The Production of Serum Unable to Support the Multiplication of Connective Tissue Cells.

Many sera throw down a precipitate after storage for two or three weeks. This precipitate is usually removed by centrifugation or Seitz filtration before stored sera are used for the cultivation of tissue cells. Horse and bovine sera produce a very copious deposit which may continue to form for some weeks. It was noticed that during storage horse serum gradually lost its capacity to support the multiplication of connective tissue cells. When tested within a few days of being drawn horse serum supported an approximately 3-fold increase in cell numbers. As in rabbit serum (Harris, 1955), the growth rate was not logarithmic but began to fall from the outset and continued to do so until growth ceased. After the horse serum had been stored for 4–5 weeks it failed to support either the multiplication of the connective tissue cells or their survival for more than a few hours. In this deficient serum the cells remained alive without multiplying for about four hours and then gradually disintegrated. All the cells were dead within 18 hours. The multiplication of the cells in fresh horse serum and their failure to survive in horse serum stored for 5 weeks are shown in Fig. 1. Bovine serum was also found to lose its capacity to support cell multiplication on storage, but a completely deficient rabbit serum could not be produced in this way.

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Restoration of the Capacity to Support Cell Multiplication by the Addition of L-*cysteine or* L-*cystine.*

It was thought that the deficiency produced in horse and bovine serum by storage might be due to the loss by degradation or precipitation of one or more essential growth factors. It was therefore decided to investigate whether the capacity to support cell multiplication could be restored to the serum by the addition of substances usually presumed to be necessary for cell multiplication.

The L-amino acids were tested first. Each of the following L-amino acids was added singly to the serum to make a final concentration of 0·001 M : glycine,

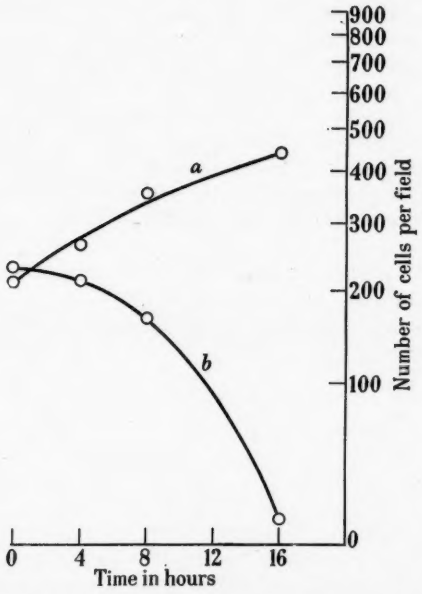


FIG. 1.—The multiplication of the connective tissue cells in fresh horse serum (*a*), and their failure to survive in stored horse serum (*b*).

alanine, valine, leucine, isoleucine, proline, arginine, histidine, lysine, glutamic acid, aspartic acid, serine, threonine, tyrosine, tryptophane, phenylalanine, methionine and cysteine. Because of its low solubility L-cystine was tested at a final concentration of 0·0001 M. It was found that the addition of either L-cysteine or L-cystine in the concentrations mentioned restored to the deficient serum its capacity to support the multiplication of the connective tissue cells. None of the other amino-acids had this effect. Fig. 2 shows a population of connective tissue cells after 16 hr. incubation in the deficient serum ; Fig. 3 shows a population of cells after 16 hr. incubation in the same serum containing L-cysteine at a final concentration of 0·001 M. The range of concentrations over which this effect could be produced was tested with L-cystine, as this is more stable in solution than L-cysteine. It was found that with a final concentration of L-cystine of

0·00002 M, the survival of the cells in the deficient serum was prolonged, but no appreciable cell multiplication took place. With a final concentration of 0·0001 M an optimal effect was obtained; when the solution was saturated with cystine (approximately 0·0005 M) the rate of cell multiplication was the same as with a final concentration of 0·0001 M. L-Cysteine, at a final concentration of 0·001 M, had the same effect as L-cystine at 0·0001 M or 0·0005 M, but at 0·01 M L-cysteine was found to be toxic.

The effect of adding L-*cysteine or* L-*cystine to fresh serum.*

In the deficient serum to which either L-cysteine or L-cystine had been added the rate of cell multiplication was found to be higher than it had been in the fresh serum. It was subsequently found that the addition of either of these amino acids to fresh serum also produced an increase in the rate and the amount of growth. In Fig. 4 the multiplication of the cells in fresh horse serum is compared with their multiplication in fresh horse serum with added L-cysteine. In serum with added L-cysteine or L-cystine any viable inoculum of cells continued to multiply until a sheet of contiguous cells was formed on the coverslip, but it has not yet been determined whether this medium will support multiplication indefinitely. The growth rate in serum supplemented with L-cysteine or L-cystine was initially linear, unlike that in serum alone, but it declined as the density of the population approached its maximum. This decline could not be prevented by changing the medium, but it remains to be shown whether it was due to some inadequacy in the medium or to other limiting factors associated with the density of the population.

The effect of adding L-*cysteine or* L-*cystine on the size of the minimum viable inoculum.*

It was previously found (Harris, 1955) that in rabbit serum it was not possible to produce growth regularly out of inocula smaller than 4500 to 5000 cells evenly distributed over the floor of the chamber, *i.e.*, about 16–18 cells per sq. mm. The minimum viable inoculum in horse or bovine serum was of the same order. The addition of L-cystine or L-cysteine in optimal concentrations to any of these sera enabled growth to occur regularly out of evenly dispersed inocula of about 4–6 cells per sq. mm. This was less than a third of the minimum viable inoculum size in serum alone.

The effect of D-*cysteine and* D-*cystine.*

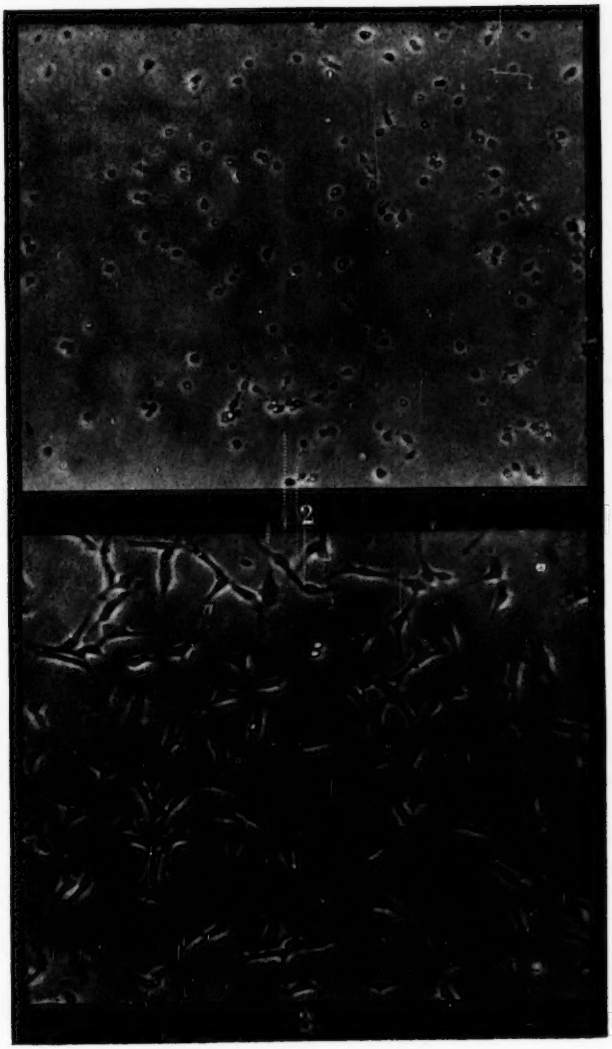
The D-amino-acids used were of the highest purity available commercially.* No concentration of added D-cystine was able to restore to deficient serum its capacity to support cell multiplication. At a final concentration of 0·001 M the

* California Foundation for Biochemical Research cfp quality.

EXPLANATION OF PLATES.

FIG. 2.—Connective tissue cells after 16 hours' incubation in stored horse serum. The cells are all dead and are disintegrating. × 64.

FIG. 3.—Connective tissue cells after 16 hours' incubation in the same serum containing L-cysteine at a final concentration of 0·001 M. The cells are multiplying. The two bright round bodies in the middle of the upper half of the field are two daughter cells immediately after mitosis. × 64.



Harris.

effect produced by D-cysteine was indistinguishable from that produced by L-cysteine, but at a final concentration of 0·0001 M D-cysteine was ineffective, whereas L-cysteine supported cell multiplication.

The effect of L-*methionine*, L-*methyl-S-cysteine*, DL-*homocysteine*, L-allo*cystathionine and glutathione.*

Since it was found that methionine was unable to restore to deficient serum its capacity to support cell multiplication, it was thought worth while to test the effects of homocysteine and cystathionine, as there is evidence that these substances may be intermediates in the transformation of methionine to cysteine

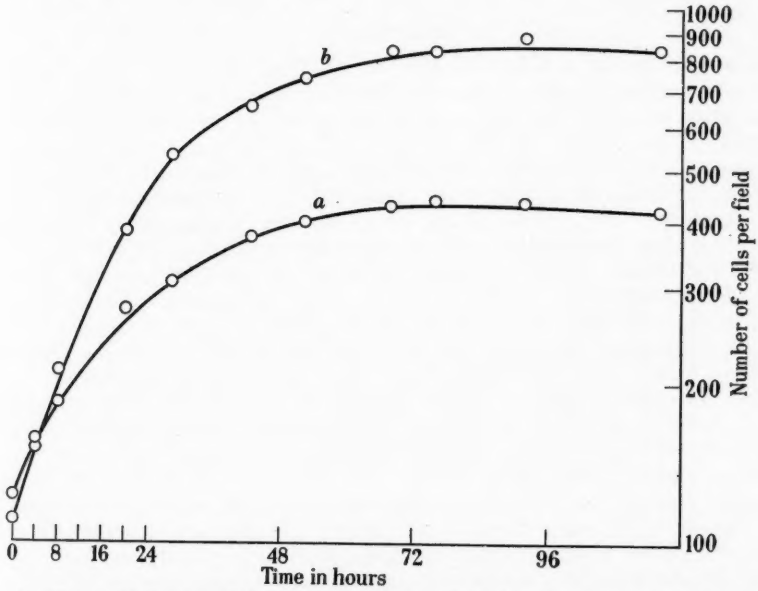


FIG. 4.—The multiplication of the connective tissue cells in fresh horse serum (*a*) compared with their multiplication in fresh horse serum containing L-cysteine at a final concentration of 0·001 M (*b*). The growth rate in the presence of added L-cysteine was initially linear. The medium in both cases was saturated with oxygen at room temperature and was changed once in each 24 hours.

in the whole animal (du Vigneaud, 1952). At final concentrations of 0·001 M and 0·0002 M DL-homocysteine completely restored the capacity to support cell multiplication, but L-*allo*cystathionine was ineffective at any concentration. L-Methyl-S-cysteine was also ineffective. L-Methyl-S-cysteine was tested together with L-cysteine, each at a final concentration of 0·0005 M, in order to see whether the methylated compound, itself ineffective, might not competitively inhibit the effect produced by L-cysteine. However, no such inhibition was detected. Glutathione, at a final concentration of 0·001 M, slightly prolonged the survival of the cells in the deficient serum, but it was unable in any concentration to restore to the serum its capacity to support cell multiplication.

The effects of thioglycollic acid, dithiodiglycollic acid, sodium sulphite and ascorbic acid.

In order to determine whether restoration of the capacity to support cell multiplication required the whole of the L-cysteine or L-cystine molecule or whether the effect could be produced by other substances containing a sulphydryl or disulphide group, thioglycollic acid and dithiodiglycollic acid were tested. At a final concentration of 0·001 M both of these substances were as effective as L-cysteine or L-cystine. Sodium sulphite was also effective at this concentration. That the activity of sodium sulphite was not simply due to its reducing power was suggested by the fact that ascorbic acid was ineffective at any concentration.

DISCUSSION.

These findings show that the survival and multiplication of rat connective tissue cells in serum depend on the availability of substances containing sulphydryl or disulphide groups. This requirement can be met by the addition of L-cysteine or L-cystine, but much less effectively, if at all, by the D-amino acids. The inefficacy of D-cystine at any concentration suggests that the effect produced by high concentrations of D-cysteine was probably due to the presence of the L-isomer as an impurity. The cystine of the serum proteins is apparently not available to the cells, since, in this form, cystine is present in large excess even in deficient serum. Reduction of the disulphide bonds of the protein does not appear to make cysteine any more readily available. This was indicated by the fact that the addition of glutathione, which could be shown to have reduced some of the disulphide bonds of the serum proteins, did not restore to deficient serum its capacity to support cell multiplication. The failure of glutathione to produce the same effect as cysteine or cystine suggests either that the tripeptide does not enter the cells freely or that it does not undergo substantial hydrolysis. It can be inferred also that methionine, methyl-S-cysteine and cystathionine are not converted to cysteine to any great extent, since these substances are unable to substitute for cysteine in restoring to deficient serum the capacity to support cell multiplication. It has recently been shown (Fromageot, 1955) that certain animal tissues are able to utilise added sulphite to form sulphinylpyruvic acid, the latter being converted to cysteinesulphinic acid in the presence of glutamate. It is possible that some such reaction might be responsible for the efficacy of sulphite and thioglycollic acid in the present experiments.

SUMMARY.

By the use of quantitative methods previously described it was found that the survival and multiplication of rat connective tissue cells were dependent on the availability of substances containing sulphydryl or disulphide groups. When the cells were cultivated in serum their multiplication was limited by the low concentration of free cysteine or cystine in the medium, the cystine of the serum proteins being apparently unavailable to the cells. The requirement could be met by adding L-cysteine and L-cystine to the medium, but much less effectively, if at all, by adding the D-isomers. L-Cysteine or L-cystine could be replaced by thioglycollic acid, dithiodiglycollic acid or sodium sulphite, but not by methionine, methyl-S-cysteine, cystathionine, or glutathione.

It is a pleasure to acknowledge my indebtedness to Sir Howard Florey, F.R.S., in whose department this work was carried out. Thanks are also due to Mr. G. J. Wren for technical assistance and to Mr. B. H. Glass for some help with the photography.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. V. THE SPECIFIC TOXIN PRODUCED BY *B. ANTHRACIS IN VIVO*.

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In previous work (Smith, Keppie and Stanley, 1953*a* ; 1953*b* ; Keppie, Smith and Harris-Smith, 1953) we were unable to kill guinea-pigs with products of *B. anthracis* grown *in vivo*. Thus, a mixture of plasma (approximately 1 part) and peritoneal and thoracic exudate (approximately 5 parts) was collected from guinea-pigs dying of anthrax. It was found that large volumes of this plasma-exudate mixture and extracts of the organisms from it were non-lethal when injected intraperitoneally in guinea-pigs. Intradermal injections of the plasma-exudate did however produce the small oedematous lesion characteristic of such material (Sobernheim, 1931 ; Watson, Cromartie, Bloom, Heckly, McGhee and Weissman, 1947). It was concluded that the products of *B. anthracis* growing *in vivo* were not markedly toxic. This agreed with the fact that large numbers of the organism (*ca* 1×10^9 chains per ml.) grew in the blood of a guinea-pig before death. It was suggested that death followed the action of harmful effects which were weak relative to the number of organisms producing them and that one such effect might be oedema production.

Identification of secondary shock as the major factor in death of guinea-pigs from anthrax (Smith, Keppie, Stanley and Harris-Smith, 1955) focussed attention on this oedema-producing factor as the primary harmful product of *B. anthracis*. It could produce the vascular damage which leads to secondary shock. The distribution and activity of this factor in the tissues of guinea-pigs dying of anthrax were therefore re-examined.

This paper describes the investigation leading to the demonstration of a specific lethal toxin produced by *B. anthracis*. Some properties of the toxin are described together with proof of its specificity. The pathological changes produced by the toxin in guinea-pigs have been studied and a connection has been established between the toxin and protective antigens of *B. anthracis* (Keppie *et al.*, 1953 ; Wright, Hedberg and Slein, 1954 ; Belton and Strange, 1954). Summaries of some of this work have already been published (Smith and Keppie, 1954 ; Smith, Keppie, Ross and Stanley, 1954*a*).

METHODS.

Collection of Body Fluids and Tissue Extracts.

When a supply of exudate was required the thoracic and peritoneal cavities of guinea-pigs (700 g. $\pm$ 25 g.) were infected with *B. anthracis* (strain N.P.) as previously described (Smith *et al.*, 1953*a*). If the aim was to achieve maximum invasion of the bloodstream and not the production of exudate in the cavities, inoculation was intradermal (Keppie, Smith and Harris-Smith, 1955).

Plasma.—Immediately the animals died, heart blood (8–10 ml.) was collected and heparinised (10 i.u. per ml.). After centrifugation the plasma was filtered through " Millipore " filters (Smith *et al.*, 1953*a*). All manipulations were carried out at 0–4°.

Thoracic exudate.—Treated as the plasma. It contained on the average 20 per cent blood.

Peritoneal exudate.—Contained practically no blood.

Lysed red cells.—One volume of packed red cells lysed with two volumes of water and filtered.

Urine.—Collected from several animals, pooled and filtered.

Spleen extract.—Ten spleens from infected guinea-pigs were ground with sand and extracted with saline (10 ml.). The extract was then centrifuged and filtered.

Extracts of in vivo*-grown* B. anthracis.—The organisms separated from three 100 ml. samples of plasma were treated thus: they were suspended, unwashed, in water (15 ml.). Streptomycin (1000 μg.) was added to one portion (A) which then stood overnight at 0°. A second portion (B) was similarly treated with streptomycin and shaken overnight at 0° with half its own volume of small glass beads (" ballotini "). In contrast to samples A and B, which were not filtered before assay in animals, a sample C was mixed with " ballotini ", shaken overnight, and filtered through " Millipore " filters. Finally, NaCl was added to all samples to isotonicity.

Biological Assays.

Skin test for oedema-producing activity.—Materials and saline dilutions of them were injected intradermally in volumes of 0·2 ml. into shaven guinea-pigs. After 18 hr. the diameter of the oedematous area and the thickness of the skin-fold were measured. In recording the results, a lesion having a fold-thickness of 8 mm. and a diameter of 40 mm. is given as 8 : 40.

Test for lethality.—Mice (18–22 g.) were injected intravenously. Volumes greater than 0·5 ml. were given in two injections separated by a few hr. Guinea-pigs (250 ± 25 g.) were injected in the saphenous vein using a local anaesthetic. Volumes greater than 5 ml. were given in two spaced injections. If the volume to be injected was more than 10 ml. for guinea-pigs or 1 ml. for mice, the plasma was freeze-dried, reconstituted and injected at twice the original concentration. All samples were centrifuged to remove gross particles. Results are reported as deaths (numerator) occurring in groups (denominator) of animals during 7 days' observation.

Chemical and Haematological Investigations.

The analytical and other methods used for diagnosing shock in guinea-pigs have been described by Smith *et al.* (1955).

RESULTS.

The Distribution of Oedema-producing Factor in Infected Guinea-pigs.

Table I summarises the results of skin tests for oedema-producing activity in body fluids and tissue extracts of guinea-pigs dying of anthrax. The values are the average from at least two similar experiments. The factor is found mainly

TABLE I.—*The Distribution of an Oedema-producing Factor in Guinea-pigs Dying from Anthrax.*

Material.	Quantity equivalent to one guinea-pig (700 g.). (ml.)	[Skin lesion (0·2ml.)]				
		Undil.	1/3.	1/9.	1/27.	1/81.
Plasma	18	9 : 40	7 : 30	6 : 25	5 : 20	4 : 15
Peritoneal exudate	10	6 : 20	*Nil*	*Nil*	*Nil*	*Nil*
Thoracic exudate	15	6 : 30	5 : 20	5 : 15	*Nil*	*Nil*
Urine	5	*Nil*	—	—	—	—
Extracts of spleen	1	5 : 30	4 : 15	*Nil*	*Nil*	*Nil*
Lysed R.B.C.	50	*Nil*	—	—	—	—
Bacterial extracts	3					
A		4 : 15	*Nil*	*Nil*	—	—
B		4 : 15	*Nil*	*Nil*	—	—
C		4 : 15	*Nil*	*Nil*	—	—

in the plasma and it is predominantly an extracellular product of the organism since extracts of the organisms, prepared by methods which had no effect on the oedema-producing activity of the plasma, had negligible action.

The activity of plasma collected at progressive stages in the final bacteraemia (Keppie *et al.*, 1955) increased with the bacterial count. Lesions in size 3 : 10, 5 : 15, 6 : 25, 8 : 30 and 10 : 40 were obtained in the intradermal test with plasma of guinea pigs containing 0·01, 1·5, 6, 24 and 100 $\times$ 10^7 bacterial chains per ml. of blood respectively.

The Lethal Effect of the Plasma of Guinea-pigs Dying of Anthrax.

The plasma containing the oedema-producing factor was tested for toxicity in mice and guinea-pigs. Control animals received the appropriate volumes of normal guinea-pig plasma with the addition of an amount of lysed red cells equivalent to the 10–15 per cent haemolysis usual in the plasma of guinea-pigs dead from anthrax.

TABLE II.—*The Lethal Effect of the Plasma of Guinea-pigs dying of Anthrax.*

	Mice.				Guinea-pigs.		
	Intravenous.		Intraperitoneal.		Intravenous.		
Vol. (ml.).	Toxic plasma.	Normal plasma.	Toxic plasma.	Normal plasma.	Vol. (ml).	Toxic* plasma.	Normal plasma.
1·0	148/150	0/15	11/16	0/5	10	12/12	2/27
0·5	64/75	0/5	8/15	0/5	6	6/10	0/3
0·25	11/50	0/5	1/15	0/5	3	1/6	—

* Deaths occurring later than the first 12 hr. after injection.

Table II summarises the results with numerous batches of plasma from infected guinea-pigs; a lethal effect is clearly demonstrated. The approximate LD_{50} of the plasma for mice and guinea-pigs was 0·35 ml. and 5 ml. respectively. A higher death rate and a shorter survival time show that the plasma was more active when given intravenously than by the intraperitoneal route which had been used in previous studies (Keppie *et al.*, 1953).

No mice were killed when batches of 5 were injected intravenously with anthrax peritoneal exudate (1 ml.), thoracic exudate (1 ml.), splenic extract (0·5 ml.) and the three bacterial extracts (1 ml.).

The Specificity of the Oedema Production and Lethal Effect.

It was important to establish that the toxic effects of the plasma were directly attributable to *B. anthracis*. The specificity was proved when anthrax antiserum neutralised both the lethal activity and the power to evoke local oedema. Two types of anthrax antiserum were used: (1) hyper-immune horse serum prepared against the non-capsulated avirulent "Sterne" strain of *B. anthracis*; (2) hyper-immune guinea-pig serum against the virulent N.P. strain of *B. anthracis*. The guinea-pigs had been primarily immunised with *in vivo*-produced protective antigen (Smith, Zwartouw and Gallop, 1954*b*) or by means of an aborted infection with strain N.P. Normal serum from horse or guinea-pigs did not have any neutralising effect.

TABLE III.—*Specific Neutralisation of the Oedema Production and Lethal Effect.*

Material.	Skin-test (undiluted plasma*).	Mice. Intravenous. 1 ml.	Intravenous. 0·5 ml.	Intraperitoneal 1 ml.	Intraperitoneal 0·5 ml.
A. Toxic plasma	10 : 40	25/25	23/25	11/15	8/15
B. A + normal horse serum†	10 : 35	10/10	11/15	—	—
C. A + horse anthrax antiserum†	*Nil*	0/15	0/15	0/15	0/15
D. A + normal guinea-pig plasma‡	10 : 40	3/3	3/3	—	—
E. A + guinea-pig anthrax antiserum‡	*Nil*	0/9	0/3	—	—

* Mean of similar results from three batches.
† One-third to one-half the volume of the plasma.
‡ One-fifth the volume of the plasma.

The results (Table III) of such neutralisation tests on a number of batches of toxic plasma show the complete specificity of the reaction in mouse toxicity tests and in the skin test. Neutralisation of the lethal action of the plasma could also be demonstrated in guinea-pigs. However, the quantitative aspect of these latter tests was marred by the death of some of the guinea-pigs given neutralised plasma intravenously. The deaths, arising seemingly from a peculiar sensitivity of guinea-pigs to the plasma, occurred immediately after injection or in a few cases within the succeeding 12 hr. The degree to which anthrax plasma was troublesome in this respect varied from batch to batch. Two out of seven batches proved to be unusable, but valid evidence of specific neutralisation was obtained with the remaining batches. Guinea-pigs were given toxic plasma (10 ml.) plus either normal plasma (2 ml.) or antiserum (2 ml.). Out of 18 animals given the unneutralised plasma 6 died within 12 hr. and the remainder from 1–4 days after injection. In contrast, out of 21 animals given neutralised plasma, 7 died within 12 hr. and the remainder survived. The deaths occurring after 12 hr. in groups of 10 animals given a test dose of 6 ml. plasma were 6 with unneutralised material and none after neutralisation.

The nature of the non-specific rapidly-acting substance in the toxic plasma which is lethal to guinea-pigs is unknown, but it is non-dialysable and is without effect in mice. In fact, 2-ml. amounts of the two batches of neutralised plasma which were unusable in guinea-pigs, when given intravenously to mice proved harmless.

Some Properties of the Toxic Plasma.

The method of collection and separation of the toxic plasma was varied to see if there was any gross loss of activity in the method described above. However, the following treatments designed to stop the action of bacteria and of enzymes produced no significant difference in the skin reaction of the toxic plasma or in its lethality to mice: (1) the blood was collected quickly into ice-cold containers containing heparin; the blood cells and bacteria were removed quickly by centrifugation and the plasma filtered immediately at 0°; (2) the same as (1) with the addition of streptomycin (80 μg./ml.) to the infected blood; (3) the same as (1) with the addition of NaF (0·01 M) to the infected blood; (4) the same as (1) with the addition of both streptomycin and NaF.

TABLE IV.—*Stability of the Toxic Plasma at 0° and after Freeze-drying.*

Treatment	Storage (wk.).	Skin test. Undil.	1/3.	1/9.	1/27.	1/81.	Mouse lethality test. 1 ml.
Fluid at 0°	0	8 : 40	7 : 28	5 : 27	6 : 18	4 : 15	20/20
	3	7 : 37	6 : 25	5 : 20	5 : 15	tr.	8/15
	6	6 : 35	5 : 25	4 : 15	*Nil*	*Nil*	4/10
	11	6 : 25	5 : 16	tr.	*Nil*	*Nil*	1/15
Freeze-dried at 0°	0	9 : 32	7 : 25	6 : 22	6 : 17	4 : 15	10/10
	3	8 : 40	6 : 28	5 : 23	5 : 20	4 : 15	5/5
	6	9 : 40	6 : 27	4 : 20	5 : 17	3 : 15	5/5
	11	12 : 40	10 : 30	6 : 27	7 : 20	5 : 18	5/5

Stability during storage.

Table IV summarises toxicity tests carried out during a period of 11 weeks on batches of toxic plasma which were stored at 0° as fluids (4 batches), and also in the dried form (6 batches). Whereas the toxicity of the fluid plasma diminished steadily, that of the dried plasma was satisfactorily preserved. The addition of NaF (0·01–0·04 M) to the fluid plasma did not prevent deterioration.

When the toxic plasma was stored in the fluid state a precipitate which was inactive in the biological tests separated slowly at 0°, and rapidly at 20–37°. A similar precipitate remained insoluble when the freeze-dried material was reconstituted. These precipitates were removed before the tests shown in Table IV were made.

Heat lability.

When two batches of toxic plasma were heated at 60° for 30 min. they completely lost both their power to produce oedema and their lethality to mice. At 37° a slower loss of toxicity occurred. A typical batch of toxic plasma such as the control material in Table IV was incubated at 37°. After 4 hr. 1 ml. killed only 4/5 mice and in the skin test the lesions at the respective dilutions noted in Table IV were reduced to 6 : 35, 5 : 28, 4 : 25, 4 : 17 and 0. After 8 hr., 0/5 mice were killed by 1 ml. and the skin reactions were correspondingly reduced, being 5 : 25, 0, 0, 0, 0, respectively. A similar result was obtained with a second batch of toxic plasma. Even more rapid detoxification occurred if the plasma was incubated at 37° after it had been separated from the blood cells but still contained large numbers of *B. anthracis*. Each of two batches of toxic plasma after 4 hr. under these conditions completely lost its ability to kill mice or to produce oedema.

Effect of aeration and shaking.

No loss of toxicity was detected when batches of plasma were shaken vigorously in thin layers at 0° for 24 hr.

The oedema-producing activity of the toxin.

Examination of the oedematous wheal which slowly developed over a period of 24 hr. after intradermal injections showed that the toxin caused vascular damage leading to oedema and haemorrhage. These changes could clearly be seen by the naked eye in the deeper vascular area of the corium. No break occurred in the surface of the skin and within 7 days all trace of the reaction had disappeared.

Microscopically, changes were found in the whole thickness of the skin down to the cutaneous muscle. No specific damage appeared to be produced in the collagen fibres or in muscle. After 24 hr. the collagen fibres were separated by oedema fluid. Tissue cells and fibroblasts, etc. were swollen. Scattered polymorphonuclear leucocytes were present most frequently in the deeper layers just superficial to the cutaneous muscle. Extravasation of red cells occurred and appeared to be related to the formation of mural thrombi in the venules of the plexus of vessels in the deep layers of the corium. This relationship was more clearly seen when a relatively deep intradermal injection was given. Fibrin formation in the tissue spaces was associated with the extravasation of red cells.

Stability of the toxic plasma at various pH levels.

It was essential for chemical fractionation to know the pH range over which the toxin could be considered stable. Samples of toxic plasma (pH 7·5) were adjusted to pH 5, 6, 9 and 10 and left for varying periods at 0°. They were then readjusted to pH 7·5 and their tissue-damaging activity and lethality in mice compared with those of a control sample. Table V summarises the results of two such experiments on different batches of toxin. It is obvious that any departure from pH 7–8 results in loss of activity which is most marked at pH 5 and 10.

TABLE V.—*Effect of Changing the pH of Toxic Plasma.*

pH.	Duration of exposure (hr.).	Skin test.					Mouse lethality test: 1 ml.
		Undil.	1/3.	1/9.	1/27.	1/81.	
7·5	Control	9 : 42	8 : 28	5 : 25	4 : 18	4 : 12	29/29
5	3	7 : 45	6 : 35	4 : 30	3 : 25	tr.	8/11
	24	7 : 40	6 : 27	4 : 26	4 : 18	„	7/10
	48	6 : 35	5 : 25	4 : 18	4 : 10	*Nil*	1/14
6	3	8 : 42	7 : 28	5 : 22	5 : 18	tr.	7/8
	24	8 : 42	7 : 27	6 : 25	5 : 17	„	8/10
	48	8 : 42	7 : 27	5 : 21	4 : 17	„	8/10
9	3	8 : 43	7 : 27	5 : 20	5 : 15	„	8/10
	24	8 : 45	7 : 25	5 : 20	4 : 15	„	8/10
	48	7 : 45	6 : 26	4 : 20	4 : 15	*Nil*	4/10
10	3	7 : 40	7 : 25	5 : 18	4 : 20	tr.	4/10
	24	8 : 35	6 : 22	4 : 15	tr.	*Nil*	5/10
	48	7 : 35	5 : 22	4 : 12	„		3/10

Ultrafiltration and dialysis.

When two batches of toxic plasma were ultrafiltered through a collodion membrane, the filtrates failed to cause oedema or kill mice. Toxic plasma was also dialysed against distilled water for 3–4 days at 0°. No significant loss of toxicity was revealed by skin tests or mouse tests on the dialysates which had been made isotonic with NaCl. However, if dialysis was made against distilled water whose pH (5–6) had not been adjusted to 7·5, a considerable loss of toxicity occurred.

Fractionation of the toxic plasma.

Many attempts have been made to fractionate the toxic plasma using a low temperature, barium acetate/ethanol method by means of which polyglutamic acid, and a protective antigen/agressin fraction were separated from plasma exudate (Smith *et al.*, 1954*b*). All were unsuccessful and resulted in a marked loss of toxin. Remnants of tissue-damaging activity were however always found in the one fraction that contained the agressin and protective antigen.

TABLE VI.—*Fractionation of Toxic Plasma.*

Fraction.	Essential nature.	Volume of yield relative to the volume of starting material.	Skin test.						Mouse lethality test.	
			Vol. injected. ml.	Undil.	1/3.	1/9.	1/27.	1/81.	Vol. injected ml.	Result.
Toxic plasma	—	—	0·2	9 : 40	7 : 30	6 : 26	5 : 20	4 : 15	1·0	10/10
Dialysate	—	1·25	0·25	9 : 37	7 : 28	6 : 24	5 : 17	tr.	1·25	10/10
2 per cent barium acetate/5 per cent ethanol fraction	Polyglutamic acid	0·2	0·2	0	0	0	0	0	1·0	0/8
2 per cent barium acetate/15 per cent ethanol fraction	The protective antigen and agressin	0·2	0·2	7 : 28	5 : 20	4 : 16	0	0	1·0	0/16
Soluble from above	Contaminating plasma protein	1·5	0·4	0	0	0	0	0	1·5	0/8

Table VI summarises the results of one fractionation. Results of assays for agressin and protective antigen (Keppie *et al.*, 1953) are not included because they were similar to those already reported for plasma/exudate (Smith *et al.*, 1954*b*).

The Relation of Toxin to Protective Antigen and Capsular Substance.

The demonstration of a specific lethal factor in the plasma of guinea-pigs dying of anthrax raised the question of its relation to a now well-established protective antigen (Keppie *et al.*, 1953 ; Wright *et al.*, 1954 ; Belton and Strange, 1954) and to the capsular substance of *B. anthracis*.

Whereas the toxin kills mice and guinea-pigs and forms a large oedematous lesion in the skin of the guinea-pig, the protective antigen produced *in vitro* by Belton and Strange (1954) is non-lethal and produces no skin reaction. Thus, 4 rabbits were unharmed by the intravenous injection of 50 ml. of antigenic filtrate prepared by our colleague F. C. Belton from cultures of the N.P. and Sterne strains. Even when tenfold ultraconcentrates of these filtrates were prepared, they proved harmless to mice (1 ml. intravenously) and provoked no reaction in guinea-pigs (0·2 ml. intradermally).

The following evidence shows that the toxin and the protective antigen are related.

(1) Hyper-immune serum prepared in the horse (Belton and Strange, 1954) against the protective antigen obtained from *in vitro*-grown cultures of a non-capsulated avirulent strain of *B. anthracis*, both inhibits the production of oedema by toxic guinea-pig plasma and abolishes its killing power. In three tests, successive batches of toxic plasma (0·2 ml.) produced their oedematous skin reaction when mixed with normal horse serum (0·1 ml.), but not when mixed with the antiserum (0·1 ml.) described above. In two tests the intravenous injection of mice with 1·0 and 0·5 ml. amounts of toxic plasma killed respectively 10/10 and 10/15 animals. The same amounts of toxic plasma mixed with $\frac{1}{2}$ vol. of normal horse serum killed 10/10 and 11/15 mice respectively. No mice died when antiserum was used. Adequate controls were included.

(2) Our colleagues, D. W. Henderson and F. C. Belton (personal communication) have shown that the serum of rabbits, monkeys and humans which have been immunised with the *in vitro*-prepared protective antigen specifically inhibits the oedematous reaction produced in the skin of the rabbit by the toxic plasma. Further, they have shown that if hyperimmune horse serum is mixed with excess of the protective antigen prepared *in vitro*, it no longer neutralises the tissue-damaging activity of the plasma.

(3) The fractionation described above shows that the remnants of the tissue-damaging activity and the protective antigen appear in the same fraction.

In contrast, toxin and capsular substance appear to be unrelated. Thus, firstly, the two horse anthrax antisera described above which neutralise the toxin were derived from the Sterne uncapsulated strain of *B. anthracis*. Further, pure sodium polyglutamate (34 and 80 mg.) produced by *B. anthracis in vivo* (Smith *et al.*, 1954*b*) and the 5 per cent ethanol/2 per cent barium acetate fraction of the toxic guinea-pig plasma which contains 50–60 per cent of this material (Table VI), were non-lethal for guinea-pigs and mice respectively. No tissue-damaging activity in the skin of guinea-pigs was shown by either preparation.

Relation of Oedema Factor to Lethal Factor.

Results from the parallel assay of material in both the skin test and the mouse lethal test have agreed. This agreement was clearly shown by the tests for toxin in the several body fluids of infected animals, by the parallel diminution in the two effects during adverse conditions of storage of toxic plasma, and by the neutralisation of both reactions with specific antisera.

This latter point is demonstrated in Table VII which shows that for each antiserum the amount required to neutralise 1 ml. of toxic plasma was the same whichever effect was used as indicator.

TABLE VII.—*Comparison of Neutralising Power of Anthrax Antisera in the Skin and Mouse-lethality Tests.*

Antiserum.	Skin test: neutralising dose equivalent to 1 ml. toxin. ml.	Mouse lethality test: protecting dose for 1 ml. toxin. ml.
Hyper-immune horse H.430 ("Sterne" vaccine)	0·03*	0·03†
Hyper-immune horse H.25 (*in vitro* antigen)	0·1	0·05
Hyper-immune guinea-pig I (strain N.P.)	0·08	0·08
Hyper-immune guinea-pig II (strain N.P.)	0·08	0·08
Hyper-immune guinea-pig III (strain N.P.)	0·13	0·13

* Amount required for complete neutralisation: partial effect with 25 per cent less serum.
† Amount required for complete protection of group: partial effect with 50 per cent less serum.
The three guinea-pig sera were obtained by successive bleedings from a group of animals during a course of immunisation.

Death of Guinea-pigs in Secondary Shock after the Injection of Toxin.

Guinea-pigs were injected intravenously with toxic plasma (10 ml.) and 1–3 days later the dying animals were examined by methods described previously (Smith *et al.*, 1955) with a view to establishing the cause of death. The specificity of these observations was assured by comparing the results with those obtained from control animals which had received toxic plasma (10 ml.) neutralised with antiserum and sacrificed at the same time. Observations were made only on animals dying subsequent to the first 24 hr. after injection, because of the peculiar sensitivity of the guinea-pig to the plasma which led sometimes to early non-specific death.

The following results were obtained using 5–10 animals for each observation:

Clinical signs.—These were not recognisable until the final 12 hr. and they closely resembled those of anthrax infection. The animals exhibited general weakness, gradually sinking towards the floor of the cage, with neck extended and hind legs outstretched. Breathing was rapid and shallow and the blood circulation in the ears was feeble.

Reduced bleeding volume.—Direct heart puncture in dying guinea-pigs yielded 3·3 (S.D. 0·8) ml. of anoxic blood, whereas control animals readily yielded 9·3 (S.D. 0·5) ml. of normal blood. Reduced blood volume and blood pressure was therefore suggested in the dying animals.

Temperature.—The mean value in control guinea-pigs was 38° (S.D. 0·5). After the injection of toxin, the temperature remained slightly subnormal (32–35°) until the rapid fall during the final 6 hr. The value at death was 27° (S.D.2).

Presence of oedema.—There was colourless subcutaneous oedema ventrally at the neck and chest and an exudate in the pleural cavities. A comparison of the plasma protein N value with that of control animals (Table VIII) confirmed the loss of protein from the blood.

Blood haemoglobin values (per cent w/v).—The values for the toxaemic (6·9, S.D. 1·1) and control animals (9·0, S.D. 3·0) were much lower than those occurring in uninjected animals (15·6, S.D. 1·4). The reason for this is not clear, but it may be due to agglutination of red cells leading to their removal from the circulation. Agglutination of guinea-pig and mouse red cells by toxic plasma can be demonstrated *in vitro* : the neutralised plasma acts similarly.

Interference with phosphate metabolism.—The plasma inorganic phosphate value in toxaemic guinea-pigs is significantly raised (Table VIII) as in secondary shock.

Disturbance of electrolyte balance.—Table VIII shows that the plasma K and Mg values of the toxaemic animals are greater than those of controls. The respective Na values do not differ, a finding corresponding to that in anthrax infection (Smith *et al.*, 1955).

Renal failure.—The results in Table VIII indicate uraemia together with an abnormally high level of plasma alkaline phosphatase such as occurred in guinea-pigs dying of anthrax although freed from active infection by treatment with streptomycin (Smith *et al.*, 1955). The toxaemic animals also developed anuria.

TABLE VIII.—*Toxaemia in Guinea-pigs : Plasma Analysis.*

Analysis (mg./100 ml.) :	*Guinea-pigs (250 g.) intravenously injected with 10 ml.	
	Toxic plasma (dying).	Neutralised toxic plasma (sacrificed).
Protein N	493 (53·0)	695 (47·0)
Inorg. P.	22·5 (9·5)	6·6 (1·0)
Na	283 (12·3)	281 (15·3)
K	40·7 (13·5)	22·4 (4·0)
Mg	3·8 (1·2)	2·6 (0·7)
N.P.N.	192 (32·5)	40·9 (10·7)
Urea	347 (44·4)	57 (14·0)
Alkaline phosphatase (units of King 100 ml.)	59 (14·5)	16 (3·0)

* Results quoted are the mean from groups of 5–10 guinea-pigs, and the standard deviations are given in brackets.

The microscopical changes found in the kidneys were of the same type as those previously recognised in infected guinea-pigs (group Y), which had died in shock despite streptomycin therapy (Smith *et al.*, 1955 ; Ross, 1955). The tubular changes were however uniformly less severe.

In animals dying 24–26 hr. after the injection of a lethal dose of toxin the only lesion might be an increased affinity of the cells lining the first convoluted tubules for acid dyes such as eosin. This was associated with some degree of pyknosis of the nuclei. The brush border of the cells of the first part of the nephron was frayed and this was clearly seen in the descending loop of Henle in preparations stained by the periodic acid Schiff method. The Schiff-positive material lay fragmented in the lumen of the tubule, and in some kidneys the lumen of the descending loop of Henle was full of it. Casts in the second part of the nephron were also Schiff-positive. In the descending loop of Henle, occasionally cells showed loss of nuclear staining. This was particularly so in one animal which survived for 72 hr.

In controls given neutralised toxin the changes in the kidneys were minimal and few casts could be found.

Preparations were made to show the distribution of alkaline phosphatase. The Schiff-positive brush border of the first part of the nephron is the site of this enzyme in the kidney of normal guinea-pigs. In animals dying from toxin a diminution of alkaline phosphatase throughout the first part of the nephron was demonstrated, and in some instances patches of the cortex gave no reaction. In the descending loop of Henle the positive reaction was given by the material within the tubule and casts in the second part of the nephron or in the collecting tubules contained the enzyme.

In controls given neutralised toxin there was a slight reduction of the alkaline phosphatase in the brush border of the first part of the nephron. Casts giving a positive reaction for alkaline phosphatase occurred in the second part of the nephron and in the collecting tubules in some of these animals.

An examination of the suprarenal glands showed that the toxin caused small extravasations of the blood in the medulla and in the inner part of the cortex. Such haemorrhages did not occur in controls given neutralised toxin.

It is clear from these findings that the injection of anthrax toxin causes the death of guinea-pigs in secondary shock with its accompanying renal failure.

Attempted Treatment of Anthrax in Guinea-pigs beyond the Critical Point of the Bacteraemia.

When it was established that *B. anthracis* formed a toxin which produced fatal shock in guinea-pigs some attempts were made to counteract these effects with therapeutic measures.

Small numbers (8–12) of infected animals whose blood contained 8–90 $\times$ 10^6 chains of *B. anthracis* per ml., and which, therefore, could not be saved by the injection of antibiotic alone (Keppie *et al.*, 1955), were given additional treatment as follows: control animals; anthrax antiserum sufficient to neutralise any circulating toxin; antiserum together with saline therapy and warmth as used in the treatment of shock (Millican, Tabor and Rosenthal, 1952). The percentages of survivors in each group are respectively 0, 8 and 33. The survival rate was poor in the animals given both antitoxin and treatment for shock but it was significantly greater than in the controls. It was not thought profitable to continue attempts to cure animals at such a late stage in the disease.

DISCUSSION.

The evidence presented shows that the oedema-producing and lethal factor present in the plasma of guinea-pigs dying of anthrax is a specific product of the

growth of *B. anthracis* and leads to the death of the host. This finding was only made possible by using the host as the culture medium for the organism.

In view of the large number of organisms (1×10^9 chains per ml. of blood) growing in a guinea-pig before it dies, it might be expected that either the potency of the toxin was low or that small amounts of it were elaborated per organism. The fact that a lethal dose was contained in a relatively large amount of plasma supports this conjecture. Studies on toxin purification now in progress are expected to reveal potency on a weight basis. All evidence indicates that the two toxic effects—oedema production and lethal action—are caused by the same substance. Skin tests have frequently demonstrated a slight tissue-damaging activity in exudates from animals infected with *B. anthracis* (Sobernheim, 1931; Watson *et al.*, 1947), and also in cultures when the medium consisted of serum (Nordberg, 1951). Such tests detect low concentrations of toxin in contrast to the lethality test in which only materials containing a much higher concentration can kill mice and guinea-pigs. The finding that only the plasma of infected guinea-pigs contains a lethal concentration of toxin, explains the previous failures of others and ourselves to demonstrate lethal activity in exudates and exudate/plasma mixtures.

Recently Evans and Shoesmith (1954) have described a product from old cultures of *B. anthracis* which causes a skin lesion entirely different in nature from our characteristic oedematous reaction. This product has yet to be shown to be lethal or specifically related to anthrax infection.

A close relationship has been established between the specific toxin and non-toxic protective antigens of *B. anthracis*. On the other hand, it has been proved that the toxicity of plasma is unrelated either to the capsule of *B. anthracis* or to polyglutamic acid. These findings are at variance with those of Watson *et al.* (1947) who related their "inflammatory factor" with polyglutamic acid and not with the protective antigen which they found in another fraction of rabbit exudates.

The ability of the toxin to produce oedema agrees with the finding that guinea-pigs dying of anthrax do so in a state of shock where oligaemia results from vascular damage. That the toxin is directly responsible for the host's death in anthrax is confirmed by our study of the pathological changes in guinea-pigs following the injection of a lethal dose of toxin.

SUMMARY.

B. anthracis produces a toxin *in vivo* which is responsible for the death of the host.

The toxin is found mainly in the plasma of guinea-pigs dying of anthrax. It produces oedema in the skin, and kills mice and guinea-pigs when injected intravenously.

Oedema production and lethal effect are due to the same toxic substance. Both effects are specifically neutralised by anthrax antiserum.

The toxin appears to be in no way connected with the capsular substance of *B. anthracis* or with polyglutamic acid. There is a close relationship between it and non-toxic protective antigens.

The cause of death in guinea-pigs dying after injections of the anthrax toxin is secondary shock. The syndrome is identical with that occurring in anthrax.

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THE EFFICIENCY OF FILTRATION BY THE POPLITEAL LYMPH NODE OF THE RABBIT.

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THERE is considerable evidence that the lymph nodes of experimental animals readily allow the passage of bacteria in the lymph stream (Noetzel, 1906 ; Buxton, 1907 ; Wells and Johnstone, 1907 ; Barnes and Trueta, 1941 ; Smith and Wood, 1949) but accurate estimation of the filtering efficiency of a lymph node has seldom been made. Drinker, Field and Ward (1934) cannulated afferent and efferent lymphatics of the popliteal lymph node of the dog and perfused the node with a suspension of streptococci. They found that 99 per cent of the organisms were removed by the node and concluded that it was an efficient mechanical filter of bacteria. A discrepancy also exists with regard to filtration of red cells. Drinker *et al.* (1934) found almost complete removal of erythrocytes by the dog's popliteal node, although massage of the node caused release of cells. Courtice, Harding and Steinbeck (1953) injected erythrocytes into the peritoneal cavity of various species and showed that they were absorbed through the mediastinal chain of lymph nodes, and that nearly all reached the bloodstream *viâ* the right lymph duct or thoracic duct. Since the work of Drinker *et al.* seems to be the only attempt to assess the filtering efficiency of lymph nodes, and their results conflict with those of other workers (using qualitative methods only) we have repeated Drinker's experiments, using rabbits and slightly different experimental conditions.

METHODS.

The principle of the method was to inject a known number of bacteria or red cells into a lymphatic in the calf of a rabbit, and to determine the number appearing in the efferent lymph central to the popliteal node ; the percentage of cells removed by the node could then be calculated.

The rabbits were anaesthetised with pentobarbitone sodium, 32–40 mg./Kg. In the majority of experiments about 0·2 ml. of 1 per cent Evans Blue solution was injected into the hind foot, to display the lymphatics. In some experiments this was omitted, without significant variation in results. The efferent lymphatics from the node were approached from the medial side by cutting the thigh muscles. Two or even three lymphatics were often present, anastomosing round the blood vessels of the node. A suitable length of lymphatic was dissected from the blood vessels, using a dissecting microscope, and cannulated with a stainless steel cannula constructed from No. 27 B.W.G. tubing ; the cannula was attached to fine polyethylene tubing and both were filled with sterile heparin solution, 1000 units/ml. 0·9 per cent saline. If more than one efferent lymphatic was present the others were ligated. The dissection usually caused spasm of the artery to the popliteal lymph node, but this passed off a few minutes after the cannulation was complete. The lymph was collected in sterile tubes, which were either at room temperature or in an ice-water mixture.

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Injections were made into an afferent lymphatic of the hind leg, using a screw-driven tuberculin syringe with a No. 30 B.W.G. hypodermic needle. Dissections showed that two in particular of the lymph vessels on the medial side of the calf almost invariably ran to the popliteal lymph node, and one of these was selected. The injection volume was usually 0·05 ml., but sometimes 0·1 ml. The injections were made over 1 to 5 min.

The leg of the anaesthetised rabbit has negligible lymph flow, so flow of lymph was promoted by passive flexion and extension of the ankle joint, carried out intermittently. This involved slight movement of the soft tissues round the knee. Throughout the dissection and subsequent collection of lymph the popliteal lymph node was not touched or traumatised, and was usually not even seen since it lay deep in the popliteal pad of fat.

The four types of bacterial cell used were spores and vegetative forms of *Bacillus subtilis* (Porton " U ") and spores and vegetative forms of *Bacillus anthracis* (M.36). Spores were suspended in distilled water, and vegetative forms in tryptic meat broth. The latter were prepared by incubating spores in broth 1½–3 hr. at 37° ; no heat-resistant forms were detected in the resulting suspensions. The mean sizes of the spores (from measurements on stained films) were 1·5 μ long by 0·8 μ wide for *B. anthracis*, and 1·4 μ long by 0·8 μ wide for *B. subtilis*. The sizes of vegetative organisms were more difficult to establish, since both species form chains. The chains of *B. subtilis* are fragile and the suspensions probably consisted mainly of single organisms ; they were about 1·0 μ wide and from 2 μ to 10 μ long, and were motile. *B. anthracis* chains are more stable, the single organisms being about 1·5 μ wide and from 5 μ to 10 μ long, and the suspensions probably contained a high proportion of chains consisting of two to four organisms. Efferent lymph was cultivated on tryptic meat agar plates, using serial dilutions (Miles and Misra, 1938). Control experiments showed that when the four types of suspension were incubated with lymph from the efferent popliteal lymphatics for 15–30 min. there was no significant change in the total count, although many of the spores became heat-sensitive. The vegetative forms did not sporulate.

Red cell suspensions were obtained by diluting heparinised blood from the same rabbit with 0·9 per cent saline. Counts in lymph were made with a conventional counting chamber after 1/20 dilution with Hayem's solution. It was found reasonably simple to make separate counts of red and white cells in the same chamber.

RESULTS.

The results are summarised in the Table.

TABLE—*The Recovery of Bacteria after Passage through the Popliteal Lymph Node.*

Injection of	No. of expts.	Dose.	Lymph flow ml./hr.	Percentage recovery.
B. subtilis spores	9	339–339,000	0·8–2·8	21·4 (±14·7)
,, vegetative cells	4	150–565,000	1·6–3·7	19·0 (±12·3)
B. anthracis spores	4	910– 12,250	1·4–1·6	9·2 (± 2·0)
,, vegetative cells	4	1550–159,000	0·5–2·4	0·03 (±0·05)

The ranges of dose and lymph flow are given. The figures for recovery are means with the standard deviations.

In the early experiments samples of lymph were collected over 5-min. intervals after the injection. The greatest outflow of organisms occurred during the first 5-min. period, and recovery of bacteria was usually complete within 15 min. A typical experiment with *B. subtilis* spores gave 15 per cent recovery in the first 5 min., and 0·3 per cent in the next 5-min. period. After 10 min. the lymph was frequently sterile. In later experiments a single lymph sample was collected over the 15-min. interval after injection.

With *B. subtilis* spores, the greatest recovery was 42 per cent of injected organisms, the least was 6·1 per cent. Two results are not included ; these gave

recoveries of 0 and 0·4 per cent. In one of these the lymphatic into which the injection was made short-circuited the lymph node, passing straight to the inguinal nodes, and this was probably true of the other experiment. We have looked for an association between efficiency of filtration and the dose of organisms injected but within the dose ranges used none was found for any of the bacterial forms; for example both the largest and smallest recoveries of *B. subtilis* spores cited above were obtained with injections of 339,000 organisms, the greatest dose used.

A relationship between rate of lymph flow and efficiency of filtration was more likely. Only with *B. subtilis* spores were sufficient experiments done to test this possibility, and a correlation between the two variables was found, the correlation coefficient being 0·79, which is significant at nearly the 1 per cent level. The Figure shows the results together with the regression line. For the low rates of

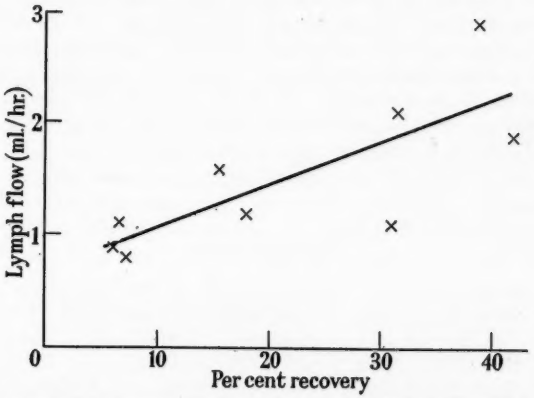

FIGURE.—The relationship between percentage recovery of *B. subtilis* spores after passage through the popliteal lymph node and the rate of lymph flow (ml./hr.). The regression line is drawn.

flow (about 1 ml./hr.) the recovery was 6–8 per cent. Allowing for the correlation between rates of lymph flow and recovery of cells there was no significant difference between the percentage recoveries of spores and vegetative forms of *B. subtilis* and of *B. anthracis* spores, but the recovery of vegetative forms of *B. anthracis* was significantly lower than that of the other three bacterial forms.

In four experiments *B. subtilis* spores were injected into an afferent lymphatic in the calf and lymph collected from the thoracic duct cannulated in the neck. In three experiments 347,000 spores were injected, and in one experiment 2670; in the former the percentage recoveries were 0·2, 1·3 and 22 per cent, in the latter 2·6 per cent. The simplest explanation is that in the experiments with low recoveries the spores passed through two or more lymph nodes, whereas the 22 per cent recovery took place through a single node. We could not establish this by dissection, but short-circuiting of lymph nodes had frequently been observed in other rabbits. It is also possible that spores sedimented in the thoracic duct giving low recoveries.

The injections of red cells were made with two dose ranges; these were necessarily higher than for bacteria, since counting the erythrocytes would have been difficult with smaller numbers. With a dose range of 10–94 $\times$ 10^6 erythrocytes there was a mean percentage recovery of 5·9 (range 2·7–14·2 in 5 experiments) in 15 min. after injection; with doses of 201–290 $\times$ 10^6 the mean recovery was 26 per cent (range 16–39 in 4 experiments). After 15 min. red cells continued to appear in the efferent lymph, but only at a few per cent per hour. Analysis of the results showed that there was a positive correlation between number of cells injected and percentage recovery; the correlation coefficient was 0·70 which was significant at below the 5 per cent level. The lymph flow varied only slightly in different experiments, and did not account for the difference in recovery of red cells.

Drinker *et al.* (1934) found that massage of a lymph node being perfused with a suspension of erythrocytes caused a release of cells. We have confirmed this in the rabbit. With the small injection doses a mean of 0·9 per cent (range 0·2–1·8) of the dose was released on firm squeezing of the node. With the higher doses a mean of 3·7 per cent (range 1·5–8·6) was released. Massage also caused a release of lymphocytes. The mean lymphocyte count (nine experiments) was 17,800 per cu. mm. (S.D. = $\pm$ 8900) in the efferent popliteal lymph before massage of the node. Massage caused a mean increase in the count of 115 per cent. In two experiments efferent lymph was collected for 1½ to 2 hr. after injection of erythrocytes (290 $\times$ 10^6 and 238 $\times$ 10^6) with total recoveries of 23 per cent and 67 per cent.

DISCUSSION.

The lymph node is usually regarded as a mechanical filter or sieve, and as such it allows particles as large as red cells to pass freely; it is therefore not surprising that, under some experimental conditions, bacteria can be transmitted through the node. The upper limit of particle size which passes through the node is not known, but removal of small bacteria and red cells must be accomplished by sedimentation in the lymph spaces as much as by sieving. The node might be regarded as a " settling chamber " (Drinker *et al.*, 1934) and this view is supported by the correlation between rate of lymph flow and percentage recovery of cells, since the efficiency of a sieve is independent of the rate of flow. Whether a cell passes through the node, is sieved out, or sediments out of the main lymph stream and is taken up by macrophages will be determined at least in part by cell shape as well as cell size. Thus the flexibility of the red cell may partly explain its ready passage through the node. On the other hand anthrax bacilli, which are relatively large, usually chained and relatively rigid, are almost entirely held up. Buxton (1907) implied that anthrax bacilli did not pass through the mediastinal lymph nodes, unlike typhoid and coliform bacilli. A further consideration is that organisms such as *B. subtilis* may be less efficiently removed owing to their motility; this may explain why they were no better filtered than bacterial spores, in spite of being larger.

A rapid lymph flow may hinder removal of cells both by interference with the sedimenting process, and by opening lymphatic channels in the node. We believe that the rates of lymph flow in our experiments were within the physiological range. The effect of the massage used was no greater than would result in a rabbit sitting quietly with occasional movements; the injections were made

slowly and the lymphatic into which the injection was being made was usually distended less than when the leg was massaged.

Our results with bacteria should be compared with those of Drinker *et al.* (1934). They used streptococci and found a 99 per cent removal by the dog's popliteal node. They did not report bacterial size or the degree of chaining, but each group of organisms would be larger than bacterial spores and would be non-motile. The low recovery of streptococci might be due to several factors. They used high concentrations of bacteria, up to 6×10^8 per ml., and this might result in " clogging " the meshes of the node and more efficient filtration ; it is perhaps significant that in their only reported experiment with low bacterial counts (300,000/ml.) there was considerably greater passage of organisms through the node. Also the experiments were continued for several hours, and within this time a cellular response in the node would certainly take place. Opie (1910) concluded that the node which had shown a cellular response to infection was a more efficient filter than the quiescent node. This was based on the work of Buxton (1907) who found that after massive injections into the peritoneal cavity there was a bacteraemia which rapidly declined after 30 minutes ; at this stage the mediastinal lymph nodes through which the bacteria were passing began to show cellular changes. Smith and Wood (1949) have extended this observation. Using intradermal injections of pneumococci in rats they showed that the immediate bacteraemia found in normal animals (caused by lymphatic absorption) was suppressed if inflammation in the nodes had been induced by a previous injection of dead staphylococci. Their results could be explained partly by a decrease in movement of the inflamed leg, causing diminished lymph flow and increased absorption of bacteria from the lymph stream, a possibility which was not eliminated.

The difference in results with erythrocytes is more difficult to understand. Drinker *et al.* found almost complete removal of red cells unless the nodes were massaged ; this might be because they used lower concentrations of red cells, since in our experiments the smaller number of cells injected were more efficiently removed from the lymph. A species difference is also possible. The popliteal node of the dog is larger than that of the rabbit, and regarded as a settling chamber it might therefore be more efficient. Wells and Johnstone (1907) found that intraperitoneal injections of *Bacterium coli* and staphylococci produced only low bacterial counts in the blood of the dog, but high ones in the rabbit, which might be because the lymph node of the dog removes a higher proportion of organisms than that of the rabbit.

It is not known to what extent the removal of cells by lymph nodes in our experiments involved phagocytosis. It is suggestive that bacterial cells were no longer appearing in the efferent lymph after 10–15 minutes, while red cells continued to be discharged for an hour or more. In applying our results to naturally occurring lymph infections it must be remembered that many bacteria may travel in the lymph stream inside polymorphs and tissue macrophages, and that these may be more efficiently removed from the lymph. However, bacteria reach the blood stream readily whether injected intraperitoneally (Noetzel, 1906 ; Buxton, 1907 ; Wells and Johnstone, 1907), into the knee joint (Noetzel, 1906), or intradermally (Barnes and Trueta, 1941 ; Smith and Wood, 1949), and they probably pass through lymph nodes on the way. The lymph node is likely therefore to be an inefficient filter in naturally occurring infections as well as under more artificial experimental conditions.

SUMMARY.

The efficiency with which the popliteal lymph node of the rabbit removes bacteria and red cells from the afferent lymph has been determined; injections were made into an afferent lymphatic during collection of efferent lymph from the node.

When *B. anthracis* spores were injected a mean of 9 per cent passed through the node; *B. subtilis* spores, 21 per cent; *B. subtilis* vegetative forms, 19 per cent; and *B. anthracis* vegetative forms, 0·03 per cent.

The percentage recovery of organisms bore a positive correlation with the rate of lymph flow.

The percentage recovery of red cells depended on the number of cells injected. Means of 6 and 26 per cent were obtained with small and large numbers of cells; massage of the node caused a further release of cells.

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THE ABSORPTION OF VITAMIN B_{12} IN GASTRECTOMISED RATS.

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INVESTIGATION of Castle's intrinsic factor has long been handicapped by the lack of any means of detecting its presence other than its therapeutic effect when given, together with a source of extrinsic factor, to patients with pernicious anaemia in relapse. The recent introduction of radioactive vitamin B_{12} has permitted more extensive clinical study, for the absorption of this substance can be measured in a patient with pernicious anaemia in remission, or in a normal subject. Difficulties remain, for not all patients can be used for these studies and only a limited amount of the isotope concerned, ^{60}Co, may be given to any one subject; in consequence an animal preparation in which the effects of intrinsic factor could be examined would prove of considerable value.

In man gastrectomy causes loss of the ability to absorb orally administered vitamin B_{12}, a loss which can be made good by feeding normal gastric juice together with the vitamin (Swendseid, Halsted and Libby, 1953). It was thought that gastrectomy in animals might have a similar result, and some experiments have been done to test this possibility. Unfortunately little is known about the secretion of intrinsic factor in any animal except the pig; the gastric juice of pigs contains intrinsic factor (Landboe-Christensen and Wandall, 1953), and in gastrectomised pigs the liver loses its content of haemopoietic principle (Geiger, Goodman and Claiborn, 1937). The pig might prove a suitable animal but the practical difficulties of maintenance and the collection of faeces would be considerable. The rat was chosen for our experiments; its faeces can be collected completely in metabolism cages, and it is simple to administer vitamin or intrinsic factor preparations by a stomach tube.

EXPERIMENTAL.

Male albino rats 3–4 months old and weighing 200–220 g. were used, some being set aside as intact controls. All rats were given 7 mg. of novarsenobenzol subcutaneously to cure Bartonella infection and were subsequently kept in isolation. They were prepared for operation by fasting overnight.

Gastrectomy.

The rat stomach is divided into two well defined parts, the glandular lower half extending from the pylorus to a line which, when the stomach is empty, runs from a point on the lesser curvature 2–3 mm. from the oesophagus to about the centre of the greater curvature, and a non-glandular upper half lined with squamous epithelium. It was thought unnecessary to resect the squamous-lined part of the stomach, and the glandular part only was removed, together with approximately the first centimetre of the duodenum, which contains the main mass of Brunner's glands.

Under ether anaesthesia and with full aseptic precautions, except that gloves were not

worn, the upper abdomen was opened by a midline incision and an eyelid retractor inserted. The stomach was brought out and the vessels supplying the greater curvature from the junction of the glandular and non-glandular parts to the pylorus were ligated with fine nylon thread. Vessels supplying the pylorus and duodenum were ligated next, and then the large artery on the anterior surface of the body. As far as possible the branches to the oesophagus and non-glandular part of the stomach were preserved. Finally the stomach was turned upwards and the large vessel supplying the posterior surface was ligated as high up as possible. The omentum was then divided along the greater curvature, the duodenum cut across about 1 cm. below the pylorus, and the pylorus and duodenum cut free. After the peritoneal cavity had been packed off, the stomach was opened on the greater curvature at the junction of the glandular and non-glandular parts, and the incision was continued with scissors along the well defined glandular-squamous boundary. On the lesser curvature, where the mucosa reaches a point about 3 mm. from the oesophagus, the squamous part was carefully preserved so that the remnant of the stomach could be closed without occluding the oesophagus. For closure and anastomosis fine unbraided silk was the most suitable suture material. This was mounted on a No. 6 curved round-bodied needle as used in surgery of the eye. The stomach remnant was closed with a continuous inverting suture. Closure was begun at the top of the stomach remnant and terminated when the remaining opening was equal in size to the cut end of the duodenum. During the early stages of closure a glass rod was inserted into the oesophagus from the stomach to ensure that the opening was left patent. The distal opening in the stomach was anastomosed directly to the duodenum, using a continuous suture which inverted the cut edges. The abdomen was closed in two layers with continuous silk sutures.

Condition of rats after gastrectomy.

After operation the rats were given 5 ml. of saline and 50,000 units of penicillin subcutaneously, this dose of penicillin being given daily for a week. For the first three days after operation the rats were given only glucose water by mouth, and then gradually reintroduced to solid food. Deaths after operation were mostly from peritonitis, obstruction or haemorrhage, but in some cases an apparently healthy animal developed extreme dilatation of the remnant of the stomach and died on the first or second day after operation.

No detailed study of the blood was made in these experiments. Animals with normal haemoglobin values were selected for operation, and haemoglobin readings and blood films were made at intervals after operation. Survivors tended to develop hypochromic anaemia, and those animals which had lost much blood at operation failed to regain normal levels of haemoglobin. The anaemia was resistant to iron therapy, both by mouth and parenterally, as has been reported by Balfour, Higgins and Woods (1950).

Those rats which survived operation usually looked healthy and gained weight almost normally for several months, but they then began to decline and died in the sixth or seventh month after operation. In many cases the symptoms were those of obstruction—failure to eat, loss of weight, dehydration and regurgitation of ingested food—and it was sometimes possible to relieve these symptoms temporarily by passing a fine rubber catheter into the remnant of the stomach in order to wash it out with saline, and by giving saline subcutaneously.

Findings post mortem.

Only one rat was killed when still apparently in good health, 130 days after operation, and its tissues were fixed immediately. Microscopically the squamous epithelium of this animal's stomach was very hyperplastic and was covered by a thick layer of keratin. Here and there in the folds of keratin were masses of bacteria, and in some places there were pockets of polymorphs between the

keratin and the epithelium. The submucosa showed intense chronic inflammation and in places giant cells, which may have been associated with the stitches put into the stomach. Two ulcers penetrating to the sub-epithelial tissue were present at the oesophageal end of the stomach. To one side of one of the ulcers a remnant of a stitch could be seen, and near the base of the other were two giant cell systems, but it seems improbable, in view of the wide-spread inflammation, that these ulcers were due to the presence of unabsorbed stitches. The oesophagus showed no evidence of hyperkeratosis, but near the junction between oesophagus and stomach the epithelium gradually thickened and passed over into the greatly hypertrophic epithelium of the stomach. The junction of stomach and intestine had healed well. In the intestine the villi were well preserved and, in contrast to the state of the stomach, there was no sign of inflammation. There was a small remnant of Brunner's glands in the intestine close to the suture line.

Other animals examined post mortem died after overt illness of varying duration, and autopsies were not made immediately after death. Two of these animals showed macroscopically a large lumen at the lower end of the oesophagus.

Five rats had ulcers in the squamous epithelium of the stomach and those examined histologically showed evidence of epithelial hyperplasia and increased production of keratin, although this was not present in the oesophagus. The ulcers were associated with acute inflammatory changes and in some stomachs there was infiltration with plasma cells and macrophages. In one animal there appeared to be a remnant of Brunner's glands.

In two rats the stomach had perforated and metallic particles coming from a gnawed cage were found outside the perforation. In another a large hair ball was present.

In view of this it is difficult, in the absence of demonstrable mechanical irritation, to draw firm conclusions about the origin of the ulcers which were present in some rats.

Measurement of absorption of vitamin B_{12}.

The absorption of vitamin B_{12} was estimated by a technique similar to that introduced by Welch and Heinle (1951) for studies in man. A measured dose of vitamin B_{12}, labelled with ^{60}Co, was given by mouth, and the amount of radioactivity subsequently excreted in the faeces was measured. When normal rats are fed vitamin B_{12} in doses of 20 μg. or more, almost the whole amount is excreted in the faeces (Lester Smith, 1952; Barbee and Johnson, 1951), but experience in man suggested that if a much smaller dose were used vitamin B_{12} absorption might be studied. Preliminary results suggested that a dose of approximately 0·005 μg. of vitamin B_{12} would be suitable in that a reasonable differential between normal and gastrectomised rats might be expected, while at the same time there would be enough radioactive matter in the faeces to permit accurate counting. This dose was used throughout these experiments and gave satisfactory results, although it may not be the optimum value for such studies. The radioactive vitamin used in these experiments had an activity of 0·6 μC/μg. At the end of the experiments the residue was tested by Dr. Lester Smith, who found that it had not lost any microbiological activity.

Although the gastrectomised rats were able to take a diet of ordinary rat cake, the following diet was given to reduce the bulk of the faeces to a convenient amount: cornflour 65 g., low vitamin content casein 28 g., brewer's yeast 5 g., and salt mixture (Hubbell, Mendel and Wakeman, 1937, but with the iron content doubled), 2 g. This diet was supplemented by milk between experiments. For 24 hr. before and after feeding vitamin B_{12} the diet was replaced by a simple paste of cornflour, except for 8 hr. immediately before and after the feeding, when only water was allowed. This was an empirical arrangement intended

to permit the greatest possible absorption of the vitamin B_{12}. After the administration of the radioactive vitamin B_{12} the rats were placed in metabolism cages and kept there until radioactivity in the faeces became negligible; 8 days were usually sufficient but some experiments were carried on for 10 days. The faeces were collected for assay of radioactivity at intervals of 48 hr., care being taken to see that any faeces adherent to the cage or collecting funnel were included in the samples. Each collection was placed in a small petri dish, dried at 37° for 24 hr., and then powdered by hand in a mortar. The amount of radioactivity in the urine was too small for measurement.

Measurements of radioactivity were made by scintillation counting, using a 5 cm. sodium iodide crystal mounted on a type 6260 photo-multiplier tube operated at 800 V. (Electrical & Musical Industries Ltd., Hayes, Middlesex). This was connected to an amplifier (AERE No. 1008B) and a scaler with discriminator bias set at 10 V. This arrangement gave a background count of about 175 per min. and, with the size of sample used, an efficiency of about 18 per cent for ^{60}Co. Standards and samples of the faeces were counted in uniform thin-walled glass bottles with an internal diameter of 2·2 cm. These bottles were centred over the crystal shield by a perspex ring. The 48-hr. samples of dried and powdered faeces did not exceed 2·5 ml. in volume; they were all made up to this volume by adding some dry food mixture to the mortar before the faeces were transferred to the counting bottles. This practice also helped to prevent particles of faeces from sticking to the mortar. Background counts were made on the empty bottles before each sample was counted. Counting times were not fixed, but were carried on long enough to keep the statistical error of counting to about 2·5 per cent of the total dose, at the 5 per cent probability level.

The quantities of vitamin B_{12} used in the present experiments were very small and a system of checks was used to minimise errors. Stock solution of vitamin B_{12} was taken up by micropipette and added to 4 ml. of water to give a concentration of 0·01 μg./ml. For each experiment doses of 0·5 ml. were taken from this solution and placed in standard counting bottles. Two further quantities of 0·5 ml. were added to 2·0 ml. of water in standard bottles, and these two bottles provided radioactive standards throughout the experiment. Before administration of the vitamin all the doses and standards were counted to check their uniformity, and after administration the empty bottles were again counted, so that any residual radioactivity might be allowed for in the subsequent calculations.

The solution under test for intrinsic factor activity (or water for control animals) was given in a volume of 2·5 or 3 ml. This volume was divided into three portions; the first was given 1 hr. before feeding the vitamin, the second was incubated with the vitamin at 37° for 1 hr. before feeding, and the third was used to wash out the bottle that had contained the vitamin and was given after feeding. Feeding was done through a polythene tube with an internal diameter of 0·5 mm. which was passed into the stomach. A 14-gauge needle and a 2 ml. syringe containing the vitamin were then attached to the polythene tube and the material injected. On a few occasions, after removal of the needle, a little fluid escaped from the polythene tube; if any vitamin was lost in this way the experiment was abandoned. Finally 0·25 ml. of air was injected and the tube withdrawn. The injection of these volumes of fluid into the stomach remnant did not appear to distress the rats.

Materials tested for intrinsic factor activity.

Rat stomach extract was prepared from the glandular parts of the stomachs of freshly killed animals. The duodenum was not included. The material obtained from each rat was ground with 1 ml. of water in a Griffiths homogeniser in the cold room. After grinding the mixture was shaken for 3 hr. and centrifuged at 6000 r.p.m. The supernatant was dialysed against tap water and distilled water successively for three days. All the operations were done in the cold room, and after dialysis the extract was kept at 4° until needed. This extract was given to the gastrectomised rats in amounts of 2·5 ml. divided into three parts as described earlier.

The pig pyloric juice was obtained by collecting the secretion flowing from a fistula; a year previously pyloric juice from this pig had been shown to contain intrinsic factor active in man (Heatley, Jennings, Florey, Watson, Turnbull, Wakisaka and Witts, 1954). It was filtered through a 5/3 sintered glass filter, and the filtrate was dialysed in the same way as the rat stomach extract. Three ml. of this filtrate were given to the gastrectomised rats in the usual way. Three separate batches, collected on different days, were used.

RESULTS.

The absorption of radioactive vitamin B_{12} was measured in four groups of animals: normal rats, gastrectomised rats, gastrectomised rats given an extract of rat stomach, and gastrectomised rats given a dialysed filtrate of pig pyloric juice. The dose of vitamin B_{12} was always approximately 0·005 μg. The results obtained with normal rats are shown in Table I, and those with gastrectomised rats in Table II. In each case the figure given is the amount of vitamin B_{12} excreted in the faeces, expressed as a percentage of the dose.

Normal rats excreted on an average 66·5 per cent of the dose of vitamin B_{12} administered with a range of 52–80 per cent. This is higher than that reported in man (Callender, Turnbull and Wakisaka, 1954) after a dose of 0·5 μg., but in terms of body weight the rat dose is larger. A smaller dose of vitamin B_{12} might have been more completely absorbed but would have necessitated some loss of accuracy in counting.

TABLE I.—*Excretion of Orally Administered Vitamin B_{12} by Normal Rats.*

Rat.	Per cent of dose excreted.	Rat.	Per cent of dose excreted.
1	80	8	73
2	71	9	63
3	59	10	76
4	52	11	55
5	76	12	72
6	61	13	72
7	56	14	65

Mean 66·5. Range 52–80.

TABLE II.—*Excretion of Orally Administered Vitamin B_{12} by Gastrectomised Rats.*

Rat.	Vitamin B_{12} and water. Per cent of dose excreted.		Vitamin B_{12} and rat stomach extract. Per cent of dose excreted.		Vitamin B_{12} and pig pyloric juice filtrate. Per cent of dose excreted.		
	1st trial.	2nd trial.	Batch 1.	Batch 2.	Batch 1.	Batch 2.	Batch 3.
1	96	97	80	81	95	—	—
2	89	93	62	83	79	—	—
3	94	—	68	57	79	90	—
4	90	98	71	61	84	93	70, 69
5	89	95	51	81	76	—	93, 93
6	92	—	—	—	—	83	89
7	93	—	—	—	—	—	94
8	93	—	—	—	—	—	93
Mean	93·8 per cent		69·5 per cent		85·3 per cent		
Range	89–98		51–83		69–95		

Gastrectomised rats were first subjected to experiment 3–4 weeks after operation: by this time they were gaining weight and looked little different from normal animals. Gastrectomised rats excreted on an average 93·8 per cent of the dose of vitamin B_{12}, with a range of 89 to 98 per cent. It is evident that they had lost almost all their ability to absorb vitamin B_{12}.

When gastrectomised rats were given extract of rat stomach along with the vitamin they excreted on an average 69·5 per cent of the dose of vitamin B_{12}, with a range of 51–83 per cent; that is, absorption was restored almost to normal.

Although there was much variation in the amount excreted, the variation was no more than in normal rats.

The results obtained with the filtrate of pig pyloric juice were inconsistent. In some experiments the absorption of vitamin B_{12} was increased, but in the majority there was no evidence of intrinsic factor activity. The mean value for excretion in this group was 85·3 per cent, with a range of 69–95 per cent, a reduction which is probably not significant.

DISCUSSION.

It is clear from these results that normal rats will absorb an appreciable fraction of a small dose of vitamin B_{12} administered orally. It is also clear that resection of the glandular part of the stomach and first part of the duodenum greatly impairs the rat's ability to absorb the vitamin. This suggests that the mucosa of the rat, like that of man, secretes an intrinsic factor necessary for the absorption of vitamin B_{12}. This view is supported by the fact that normal absorption was restored in gastrectomised rats when an extract of rat stomach was fed with the vitamin. Both normal rats, and gastrectomised rats given a rat stomach extract, showed much individual variation in their ability to absorb vitamin B_{12}. We have no explanation for this variation, which is similar to that seen in man.

The results obtained when a preparation of pig pyloric juice was given with the vitamin B_{12} were inconsistent. In some rats absorption of the vitamin appeared to be increased, although never to the degree seen with an extract of rat stomach, but in others there was no detectable effect. The results do not suggest that these variations were due to different degrees of intrinsic factor activity in the three batches of juice, but other explanations can be suggested. There may have been too little intrinsic factor present in 3 ml. of this juice to produce a consistent effect, or a small dose of intrinsic factor may produce a demonstrable effect only when other unknown variables are favourable. Another possibility is that the responses were masked by vitamin B_{12} already present in the pyloric juice; Landboe-Christensen and Wandall (1953) reported that appreciable quantities of vitamin B_{12} were present in pig pyloric juice, and although it was hoped that dialysis would remove most of it there may have been enough left to reduce the absorption of the labelled vitamin. It is also possible that there are species differences in intrinsic factors, and that pig intrinsic factor may not be effective in the rat.

The inflammatory lesions found after death in the remnant of the stomach of the gastrectomised animals which were examined may have influenced their responses to feeding with the vitamin, although there was no sign of ill health at the time of the experiment. In the experiments with the extract of rat stomach any such influence was insufficient to mask the effect of intrinsic factor, but it might have been concerned in the variability of the results.

The changes in the squamous part of the stomach are of interest in relation to the observations of Balfour *et al.* (1950) on totally gastrectomised rats. These authors found that total gastrectomy was followed by sufficient hyperplasia of the squamous epithelium of the oesophagus, which is continuous with that of the upper part of the stomach, to cause obstruction. Balfour *et al.* were able to delay or prevent the development of hyperplasia by administering human gastric

juice, which they considered corrected a specific deficiency resulting from the removal of the stomach. In the one rat of the present series which was killed while still apparently healthy there was very considerable hyperplasia and hyperkeratosis of the epithelium of the remnant of the stomach, although none was seen in the oesophagus. In the stomach of three rats preserved for histological examination some hours after death there was also hyperplasia and hyperkeratosis.

With the evidence to hand it is not possible to state with any assurance what the cause of the ulceration was. It may have been contributed to by mechanical causes or it may have been due to some deficiency depending on the loss of gastric juice. Hoelzel and da Costa (1937–38) ascribed ulceration of the squamous part of the stomach of intact rats to the effects of starvation, a low protein diet or a diet deficient in essential amino acids. In the present series of experiments ulceration occurred in the absence of gastric juice. The phenomenon seems worthy of further investigation.

The method here used did not prove satisfactory for the assay of pig pyloric juice for intrinsic factor; attention has been drawn to possible reasons for this. On the other hand the results obtained with extracts of the animals' own gastric mucosa were satisfactory, and the technique, or adaptations of it, might be of use for the examination of problems relating to the mode of action of intrinsic factor, to its quantitative relationships with vitamin B_{12}, and to the effects of systemic or intestinal disturbances on the absorption of vitamin B_{12}.

SUMMARY.

When normal rats were fed 0·005 μg. of vitamin B_{12} labelled with ^{60}Co the average amount excreted in the faeces was 66·5 per cent of the dose.

After the same dose of labelled vitamin B_{12} gastrectomised rats excreted an average of 93·8 per cent of the dose.

When given an extract of rat stomach along with the vitamin B_{12} the gastrectomised rats excreted an average of 69·5 per cent of the dose.

A dialysed filtrate of pig pyloric juice did not consistently reduce the amount of vitamin B_{12} excreted.

Hyperplasia and hyperkeratosis were observed in the squamous epithelium of the remnant of the stomach. Ulcers, the cause of which is not clear, were also encountered.

(Since this paper was written Latner (1955) has mentioned unpublished observations on normal and gastrectomised rats affording evidence that "intrinsic factor" has some degree of species specificity.)

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THE SPECIFIC PRECOCIOUS PROTECTIVE ACTION OF TETANUS TOXOID.

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THE first demonstration of a specific precocious protective action of tetanus toxoid, not involving the classical antibody response, appears to have been made by Krech (1949) who gave tetanus toxoid to mice in two doses 24 and 48 hours before a lethal dose of tetanus toxin. The eight mice injected with the toxoid survived, but the two control animals, receiving toxin alone, died on the sixth and ninth days respectively. A retardation of the signs of tetanus was observed in guinea-pigs when the toxoid was administered one day before the toxin (Lemétayer, Nicol, Girard, Corvazier and Cheyroux, 1950), and the phenomenon was confirmed in mice when a single large dose of toxoid was given 24 hours before the toxin (Raynaud, Lemétayer, Turpin, Nicol and Rouyer, 1951). It was shown that if the toxoid was given earlier its effectiveness diminished until there was little effect when given 6 days before the toxin (Raynaud and Wright, 1953 ; Lemétayer, Raynaud, Nicol and Turpin, 1954). The specific nature of the phenomenon has been demonstrated by showing that diphtheria toxoid, given in a volume or Lf dose equal to a protective dose of tetanus toxoid, has no influence on the effect of tetanus toxin (Goldman, Turner and Stafford, 1954 ; Lemétayer *et al.*, 1954). The protective action of tetanus toxoid has also been shown to be greatest when the toxoid and toxin are given simultaneously or mixed *in vitro* (Goldman *et al.*, 1954). In seeking to understand its mechanism, Wolters and Fischoeder (1954) found that the attachment of tetanus toxin to brain brei was blocked by previous exposure of the brain tissue to toxoid *in vitro*. A similar effect was shown when the animals from which the brain tissue was obtained were treated 18 hours before death with tetanus toxoid.

That the effectiveness of toxoid falls off soon after its administration, and that a blocking action by toxoid can be shown *in vitro*, is strong evidence for the view that the phenomenon is not due to the rapid formation of classical antibodies. Two explanations for the precocious protective action of the toxoid are current: (1) the toxoid competes with the toxin for a hypothetical receptor substance in the brain tissue in an analogous fashion to competitive inhibition ; (2) the toxoid becomes irreversibly fixed to the receptor substance and blocks the fixation of the toxin in a manner analogous to non-competitive inhibition. Previous work has not demonstrated the first hypothesis because of the small range of doses of toxin used, but the work of Wolters and Fischoeder gives some support to the second hypothesis. There is also the possibility that the toxoid forms complexes with the toxin and thus prevents its action, for all investigators have had to use relatively very large doses of toxoid to influence the effects of small doses of toxin.

The present investigation was designed to explore the phenomenon and if possible to test the idea of competition of the toxoid with the toxin.

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MATERIALS AND METHODS.

Animals.—Healthy young white mice of an unnamed but inbred homogeneous strain were used. For one experiment C57 Black mice were used (see Table III). Their weights varied from 17–25 g., unless otherwise stated. Ten albino rabbits, 2·5–3·2 kg. were also used.

Toxins.—Tetanus toxins from two different sources were used, " CN 655 " toxin (Wright, Morgan and Payling Wright, 1950), and " Harvard " toxin (Wright, 1953). The dried toxins were dissolved in sterile saline or in sterile 1 per cent peptone in saline immediately before use. An assay of the toxins by the method of Ipsen (1941) during the course of the experiment was made on 20 g. mice and the LD_{50} found to be 0·075 μg. for CN 655, and 0·006 μg. for the Harvard toxin.

Toxoids.—Tetanus toxoids from two different sources were used. One was prepared at the Wellcome Research Laboratories and contained 1100 Lf/ml. and the other was made at the Pasteur Institute and contained 1200 Lf/ml. (originally the Pasteur toxoid was of a higher Lf value, but much of the material had precipitated out.) It was possible to give up to 1·5 ml. (1650 Lf) of the Wellcome toxoid intravenously to a 20 g. mouse in about 5 min., but some of the animals died immediately after ; therefore, most intravenous injections were 1·0 ml. or less. Up to 0·5 ml. (600 Lf) of the Pasteur toxoid was tolerated, but several immediate deaths occurred, and the highest dose usually used was 0·3 ml. (360 Lf). Animals surviving the initial effects of the toxoids alone showed no subsequent ill effects.

Injections.—All injections into mice were made into the warmed tail veins. When both toxin and toxoid are described as being given at the same time the toxin was usually given a few minutes before the toxoid. The toxin injections into rabbits were made into the ear veins and the toxoid injections were given into the cerebrospinal fluid or medulla oblongata as previously described (Wright, 1953).

Observations.—The animals were observed at frequent intervals, especially if the period of survival was expected to be short. The time of death was taken as the mid-point between the time of the last observation and the time of finding the animal dead. Some of the animals surviving for longer periods were not observed for periods of 8 to 14 hr. during the night.

RESULTS.

Table I shows the effects of giving various doses of the toxoids at different intervals before toxin. It appears that at the higher doses of toxin the toxoid delays, but does not prevent death. Thus where 120 LD_{50} toxin was used, only 12 to 36 LD_{50} of this was apparently active (as judged by the death time) ; *i.e.*, the toxoid seemed to protect against 84–108 LD_{50}. However, neither dose of toxoid prevented death following 36 LD_{50} or more of toxin.

TABLE I.—*Mean Periods of Survival (hours) of Mice given Various Doses of Tetanus Toxoid and Tetanus Toxin (CN 655).*

Dose of toxin (LD_{50}).	Toxin alone.	0·3 ml. Wellcome toxoid 12 hr. before toxin.	1·5 ml. Wellcome toxoid 12 hr. before toxin.	0·3 ml. Pasteur toxoid 12 hr. before toxin.	0·3 ml. Pasteur toxoid 24 hr. before toxin.
1200	10·3 (3)	13·7 (6)	14·0 (3)	8·5 (2)	—
120	20 (8)	31·4 (5)	31·3 (3)	29 (3)	—
36	24 (8)	75·7 (9)	83·7 (3)	—	50 (1)
12	36·2 (10)	208 (6)	S (3)	—	129 (2)
4	53 (2)	—	—	—	192 (1) ; S (1)
2	132 (7)	—	—	—	S (4)

Figures in brackets are numbers of animals per group.
S = survival for at least 20 days.

When different doses of toxoid are administered 12 hr. before a standard dose of toxin (Table II) it is seen that as the dose of toxoid is raised in a nearly logarithmic fashion, the prolongation of life is increased in an approximately arithmetic fashion. Even if this relationship is true its biological significance is not clear.

TABLE II.—*Periods of Survival of Mice given 36 LD_{50} Tetanus Toxin (CN 655) Intravenously and Various Doses of Tetanus Toxoid (Wellcome 330 Lf/ml) Intravenously, 12 hr. before the Toxin.*

Number of animals.	Weights (g.).	Dose of toxoid (ml.).	Periods of survival (hr.).	Mean periods of survival (hr.).
6	23, 22, 21 21, 21, 21	*Nil*	24, 22, 24 25, 29, 24	25
3	20, 20, 20	0·003	52, 26, 25	34
3	20, 22, 23	0·01	29, 41, 36	35
3	20, 21, 23	0·03	53, 41, 41	45
3	20, 21, 22	0·1	90, 67, 41	66
3	24, 22, 20	0·3	64, 106, 76	82
3	24, 23, 20	1·5	71, 90, 90	84

Toxoid was given to groups of mice before, after, and at the same time as toxin (Table III). When the toxoid was given before or at the same time as the toxin, there was a considerable delay in death, but when the toxoid was given after the toxin there was no prolongation of the period of survival. It was concluded that once the toxin becomes fixed, the administration of toxoid has no effect.

TABLE III.—*Periods of Survival of Mice given 36 LD_{50} Tetanus Toxin (CN 655) Intravenously, and 0·3 ml. (330 Lf) Tetanus Toxoid (Wellcome) Intravenously before, with, or after the Toxin.*

Time that toxoid was given.	Number of animals.	Weights (g.).	Period of survival.	Mean period of survival.
12 hr. before toxin	6	21, 21, 19 19, 19, 18	76, 60, 69 90, 76, 74	72·5
At the same time as toxin	6	21, 20, 20 18, 18, 18	75, 82, 63 82, 63, 59	70·7
12 hr. after toxin	6	21, 20, 20 19, 19, 17	33, 27, 24 25, 23, 21	25·5
No toxoid	6	23, 22, 21 21, 21, 21	24, 22, 24 25, 29, 24	24·7

Because of the difficulty of interpreting the delay in death produced by the toxoid it was necessary to try to find the doses of toxoid that would just prevent death from various doses of toxin. The results, seen in the Figure, show that there is a relationship between toxin and toxoid doses which is linear if both substances are plotted on logarithmic scales. It is seen that as the dose of toxin is increased, the amount of toxoid necessary to save the animals has to be raised at a greater rate.

The results shown in Table I suggested that the Wellcome toxoid was more effective than the Pasteur toxoid, although the differing time intervals and the small number of the animals used affect the comparability of the two series. Table IV shows the results of a direct comparison of the two toxoids from which it is seen that both have approximately the same protective power as judged by delay in death time.

TABLE IV.—*Periods of Survival of Mice (C57 Black) given 80 LD_{50} Tetanus Toxin (Harvard), and either Pasteur or Wellcome Toxoid, both Intravenously at Same Time.*

Weights of animals (g.).	Type and dose of toxoid.	Period of survival and mean (hr.).	
29	*Nil*	19	18·1
31		18	
28		18	
26		16	
25		16	
27		22*	
28	360 Lf 0·3 ml. Pasteur	24	27·3
26		24	
28		26	
26		27	
24		28	
30		31	
25	330 Lf 0·3 ml. Wellcome	24	28·0
28		25	
24		27	
27		30	
29		31	
26		31	

* A small quantity of toxin escaped during injection.

In view of the exceptional vulnerability of the medullary centres of rabbits to tetanus toxin (Wright, 1953), an attempt was made to protect these animals by direct injections of toxoid into the medulla oblongata and into the cerebrospinal fluid. Tetanus toxin ($1{\cdot}33 \times 10^4$ mouse LD_{50} "Harvard") was given intravenously to 10 rabbits. Six of these received tetanus toxoid (0·025 ml. to 0·5 ml. " Wellcome ") directly into the medulla oblongata or cerebrospinal fluid at the same time or 6 hr. before the toxin. The mean survival period of the animals treated with toxoid was 120 (S.E. $\pm$ 18) hr. while the control animals showed a mean survival period of 77 (S.E. $\pm$ 12) hr. This result suggested that the toxoid had provided some degree of protection, though the number of animals used is small. It should be pointed out, however, that the risk of physical disturbance of vital structures greatly limits the amount of toxoid that can be introduced into the medulla oblongata, and the quantity used in this experiment is small when compared with that required to protect mice when the toxoid was given intravenously.

It was noticed that the mice which were only partially protected with tetanus toxoid did not show the usual signs of tetanus, but instead showed weakness, flaccidity, were limp when picked up and many showed a gross vertical tremor of their heads. When found dead they were in a curled up posture in contrast to the extensor position found in animals dying from tetanus toxin alone. These signs were particularly looked for when the two different toxoids were tested against the Harvard toxin. All the mice given toxin alone showed rigid muscles, extensor spasms, convulsions and tails that were held stiff off the ground. On the other hand the animals given toxoid in addition to toxin showed flaccid muscles, hollow flanks, absence of convulsions and muscular spasms, and limp tails that rested on the ground. Some of these partially protected animals showed a gross tremor of the front half of their bodies. The appearance of the mice dying in this fashion was closely similar to that seen in botulinum intoxication.

DISCUSSION.

The specific precocious protective action of tetanus toxoid is now well established and has been confirmed in several different laboratories with toxins and toxoids from many different sources and in several different animal species. The outstanding problem is the analysis of the mode of action of the toxoid. The original suggestion of Krech (1949) that it acts by stimulating the early formation of antibody would appear to be highly unlikely. The effectiveness of the toxoid varies inversely with the delay in giving toxin (Raynaud and Wright, 1953; Lemétayer *et al.*, 1954) until the point is reached when the production of classical antibody leads to antitoxic immunity. Efforts to find antibody during the early period have failed (Lemétayer *et al.*, 1954). The idea that the toxoid blocks the action of toxin derives some support from the findings of Wolters and Fischoeder (1954) who showed that less toxin became attached to brain brei previously treated with toxoid. Although it is not clear from their preliminary report whether pretreatment of the brain brei *in vitro* with a non-specific protein material analogous to tetanus toxoid would not have had a similar effect in preventing the adherence of tetanus toxin, they were able to produce a more striking failure of toxin adherence when the brains were obtained from animals given 150 Lf tetanus toxoid subcutaneously 18 hr. before being killed. It might be questioned whether *in vitro* studies such as these have a bearing on the effect of toxin in the intact animal, although it is known that there is a relationship between the susceptibility of an animal and the ability of its nervous tissues to fix toxin (Metchnikoff, 1898; Wassermann and Takaki, 1898; Davies, Morgan and Wright, 1955). Toxin becomes fixed rapidly to brain tissue and its effects are then not influenced by large doses of antitoxin (Wright, 1954). Similarly from our results it is clear that once the toxin has become fixed the administration of toxoid fails to alter the course of the disease. On the other hand when the toxoid is given 12 hours in advance of the toxin, the protection is no greater than when given simultaneously, a result hardly to be expected if the blocking of a receptor substance was envisaged.

If the toxoid was preventing the action of the toxin by simple combination in the blood stream, one would expect a directly proportional relationship between the dose of toxoid and toxin. This has not been found.

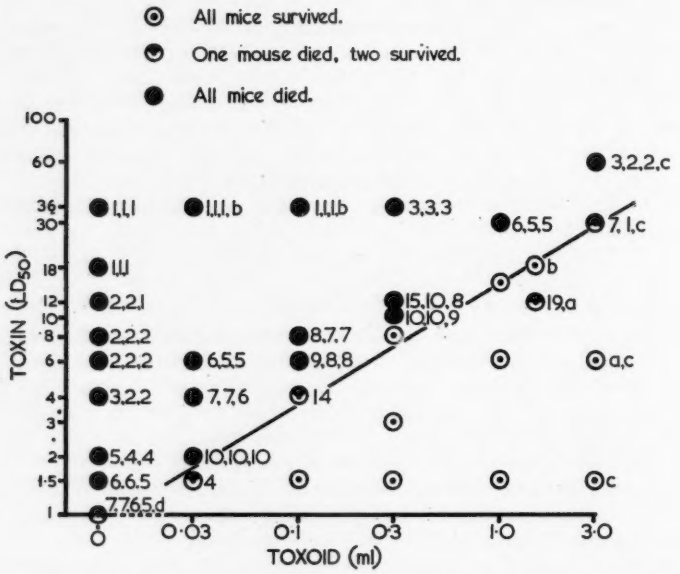
We believe that our results are consistent with the view that the toxoid molecules are competing, in a chemical sense, with the toxin molecules for some hypothetical receptor substance.

When toxoids from two different sources were administered to mice in approximately equal Lf doses, it was found that the amount of protection, as measured by the delay in death, did not differ significantly.

Pillemer, Wittler, Burrell and Grossberg (1948) have described pure crystalline tetanus toxin as containing 3600 Lf and $6{\cdot}6 \times 10^7$ m.l.d. (mouse) per mg. N. If tetanus toxoid is assumed to have as many molecules per Lf as toxin, 1 Lf toxoid would be equivalent to $1{\cdot}8 \times 10^4$ m.l.d. toxin. Our results show (see Figure) that at least 33 Lf of toxoid are necessary to protect mice from 1·5 LD_{50}. Assuming that 1·5 LD_{50} is equivalent to 1 m.l.d. and that the molecular weights of toxin and toxoid are of the same order, $6{\cdot}0 \times 10^5$ molecules of toxoid are necessary to combat one molecule of toxin. When the higher dose of 30 LD_{50} toxin is employed, a molecular ratio of $3{\cdot}0 \times 10^6$ to 1 is found. This could mean that either the toxoid was extremely inefficient in competing with the toxin, or that only a very small number of the toxoid molecules were active. The very large quantity of toxoid

necessary to protect against even small amounts of toxin makes the therapeutic use of toxoid quite impracticable. If, however, only part of the toxoid is active, it might be possible to separate a useful therapeutic agent which might act immediately and also as a classical antigen later.

When animals were only partially protected from the effects of toxin by the toxoid they showed signs of paralysis quite unlike tetanus. A paralytic action of tetanus toxin has been observed in the eye (Ambache, Morgan and Payling Wright, 1948*a*), where it would appear that toxin blocks the formation of acetylcholine (Ambache *et al.*, 1948*b*). The effect of the toxoid might be either to unmask



FIGURE—The deaths and survivals of groups of mice given various doses of toxin (CN 655) and toxoid (Wellcome). The figures to the right of the points are survival periods in days. Three mice were used for each point except (*a*) two mice (*d*) six mice. All injections were given intravenously except (*c*), where 2 ml. of toxoid was given intraperitoneally. The toxin and toxoid were given at the same time except (*b*) when the toxoid was given 12 hours before the toxin.

another, perhaps peripheral, action of the toxin, or to reveal another component of the toxin in addition to the tetanospasmin and tetanolysin. Concerning the paralytic effects of toxin and toxoid, it is interesting to note the findings of Miayamoto (1900), who observed paralytic features in his animals treated with old tetanus toxin, where much of the toxin may have been converted to toxoid.

Finally, it is perhaps relevant to note the observations of Evans and Perkins (1954) who have described in mice an early specific immunity to intracerebral infection with *Haemophilus pertussis*. This was produced by a single injection of pertussis vaccine and was not associated with demonstrable circulating antibody.

SUMMARY.

Tetanus toxoid, when given in large doses, is capable of exerting a specific precocious protective action against tetanus toxin. This action is greatest when the toxoid is given intravenously at the same time or shortly before the toxin.

The results of a quantitative study of this effect were consistent with the view that the toxoid competes with the toxin for some hypothetical receptor substance but that once the toxin has become fixed to the brain tissue the toxoid exerts no effect.

Animals whose deaths from the effects of tetanus toxin are delayed by the administration of toxoid, do not show the usual signs of tetanus, but show paralytic signs similar to botulinum intoxication.

We wish to thank Professor M. Raynaud, Pasteur Institute, Paris, and the Director, Wellcome Research Laboratories, for providing us with the purified toxoids, and Professor G. Payling Wright for his help and constructive criticism during the course of this work.

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INDUCTION OF TOLERANCE TO SKIN HOMOGRAFTS IN RATS BY INJECTION OF CELLS FROM THE PROSPECTIVE DONOR SOON AFTER BIRTH.

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IT has been shown by Billingham, Brent and Medawar (1953) that injection of living homologous cells to foetal mice and chicks affects them in such a way that, when they grow up, they are partially or completely tolerant to skin grafts transplanted from the animal that provided the inoculum (or an animal isogenic with it). Subsequently Woodruff and Simpson (1954), in a preliminary communication, have reported that a similar phenomenon occurs in rats, with the important difference that in this species injection immediately after birth confers a high degree of tolerance, whereas in mice, according to Billingham *et al.*, it usually fails to do so.

It is pertinent to ask whether the period during which exposure to foreign homologous cells results in tolerance can be extended, and in seeking to answer this question experimentally it is natural to consider first procedures which have been shown to modify the reaction of adult recipients to homografts. One such procedure, administration of cortisone, was tried in a few animals by Billingham *et al.* in their experiments with mice, but proved ineffective. It seemed to us worth while, however, to undertake a further trial in another species and on a more extensive scale, pushing the dosage almost to the limit of tolerance.

METHODS.

General Plan of the Experiment.

Twelve litters of Wistar rats, 4 new-born, 4 two weeks old and 4 four weeks old, were taken from stock, and animals in excess of 8 in each litter destroyed. Each immature rat received a single subcutaneous injection of a cell suspension prepared from the spleen of an adult black and white hooded rat. In general half the members of a litter received cells from one donor and half from another*.

Two of the litters in each age group were chosen by randomisation for treatment with cortisone, and two were used as controls. This was done because a preliminary trial showed that cortisone caused a much greater mortality if the treated animals had to compete with untreated litter-mates.

The immature Wistars comprising the twelve litters were weaned at 8 weeks, and when 16 weeks old each received a skin graft from the black and white donor whose cells it had encountered previously. At the age of 32 weeks all surviving animals with successful grafts, and a proportion of animals in which the grafts had been destroyed, received a second-set graft from the original donor. The fate of these grafts was determined by periodic inspection and by serial biopsy.

* The recipients corresponding to one of the donors were distinguished by having the tips of their tails cut off.

Preparation and dosage of cell suspensions.

Approximately two-thirds of the spleen of each adult (250–300 g.) black and white donor was removed aseptically under general anaesthesia. Bleeding was controlled with fine silk ligatures.

Cell suspensions containing approximately 180,000 cells per c.mm. were prepared by gently grinding each portion of spleen separately with 1·5 ml. saline (0·9 per cent NaCl) in a glass homogeniser. The amount of suspension injected varied according to the age of the recipient as shown in Table I.

TABLE I.—*Dosage of Cell Suspension and Hormones.*

Age and weight of rats at beginning of experiment.	Cell suspension (single injection).	Cortisone acetate (given daily for 12 days).	ACTH (long-acting) (single injection).
New-born (5–6 g.)	0·1 ml.	0·05 mg.	0·3 I.U.
2 weeks (approx. 23 g.)	0·2 „	0·20 „	0·6 „
4 weeks (approx. 50 g).	0·3 „	0·50 „	1·2 „

Hormone treatment.

Cortisone-treated animals received twelve daily subcutaneous injections of cortisone acetate suspension, the first being given just before the injection of foreign cells. On the day of the last cortisone injection each animal received in addition a single subcutaneous injection of long-acting ACTH (Organon Corticotrophin "Z"). The dose of the hormones varied according to the age of the recipient at the beginning of the experiment, as shown in Table I.

Skin grafting.

*First-set grafts.**—Under ether anaesthesia a large area of split skin was removed from each donor with a small Padgett-type dermatome, set so that the clearance between blade and drum was 0·008 in. (0·2 mm.), and cut into 2 × 2 cm. squares. The raw areas were dusted with penicillin-lactose powder, but no dressings were applied. A full-thickness defect, slightly larger than 2 × 2 cm. was prepared on each recipient by removing the skin down to the panniculus carnosus, and then covered with split-skin from the appropriate donor. Each graft was anchored with eight sutures, and stretched slightly so that it exactly fitted the defect. Biopsies were performed 2, 4, and 12 weeks after grafting.

Second-set grafts.—Second-set grafts, of the same dimensions as the first-set grafts, were obtained in the same way, after which the donors were killed. Each graft was applied to a full-thickness defect prepared on the opposite side of the thorax to that used for the first graft. Biopsies were performed 1, 2 and 4 weeks after grafting.

A detailed description of the techniques of split-skin grafting in rats has been given in a previous paper (Woodruff and Simpson, 1955).

RESULTS.

Development of Cortisone-treated Rats.

Overall growth and gain in weight were retarded for a week or two in rats treated with cortisone from birth, but by 3 weeks the treated rats had caught up with the controls. The teeth erupted earlier and the eyes opened earlier in the treated rats (Table II and Fig. 1–4). Cortisone treatment started at 2 or 4 weeks of age also retarded growth (Table II).

* We adhere to Medawar's (1944) terminology although we have used only a single graft at each operation.

TABLE II.—*Development of Cortisone-treated and Control Rats.*

Observation.	Treated.*	Untreated.†	Remarks.
Cortisone Treatment Started on Day of Birth.			
Mean weight (g.) at birth	5·6	5·6	
1 week	10·9	13·1	Difference statistically significant by t-test ($P < 0·01$)
2 weeks	22·1	23·3	Difference not significant.
3 „	36·3	36·7	
4 „	50·0	46·2	
8 „	122·0	126·3	
12 „	184·2	197·3	
Age at which teeth erupted in both upper and lower jaws (days)	5–7	9–11	
Age at which eyes opened (days)	10–12	15–16	
Cortisone Treatment Started at Two Weeks.			
Mean weight (g.) at 2 weeks	23·6	23·0	
3 „	29·5	31·9	Difference not significant.
4 „	34·9	48·0	Difference statistically significant by t-test ($P < 0·001$).
5 „	58·0	62·0	Difference not significant.
6 „	84·3	83·9	
8 „	125·6	128·5	
Cortisone Treatment Started at Four Weeks.			
Mean weight (g.) at 4 weeks	49·0	54·2	Weekly weight gain significantly greater in control animals from 4 to 6 weeks.
5 „	59·0	73·2	
6 „	67·6	92·9	
7 „	92·1	114·3	Weekly weight gain significantly greater in treated animals from 6 to 8 weeks.
8 „	108·4	125·4	
12 „	141·5	163·0	

* For dosage see Table I
† No hormones.

Behaviour of the Skin Homografts.

The behaviour of the skin grafts is summarised in Tables III and IV, and illustrated in Fig. 5–15. As might be expected in an experiment extending over months and involving many operations in each animal, there were deaths. Fortu-

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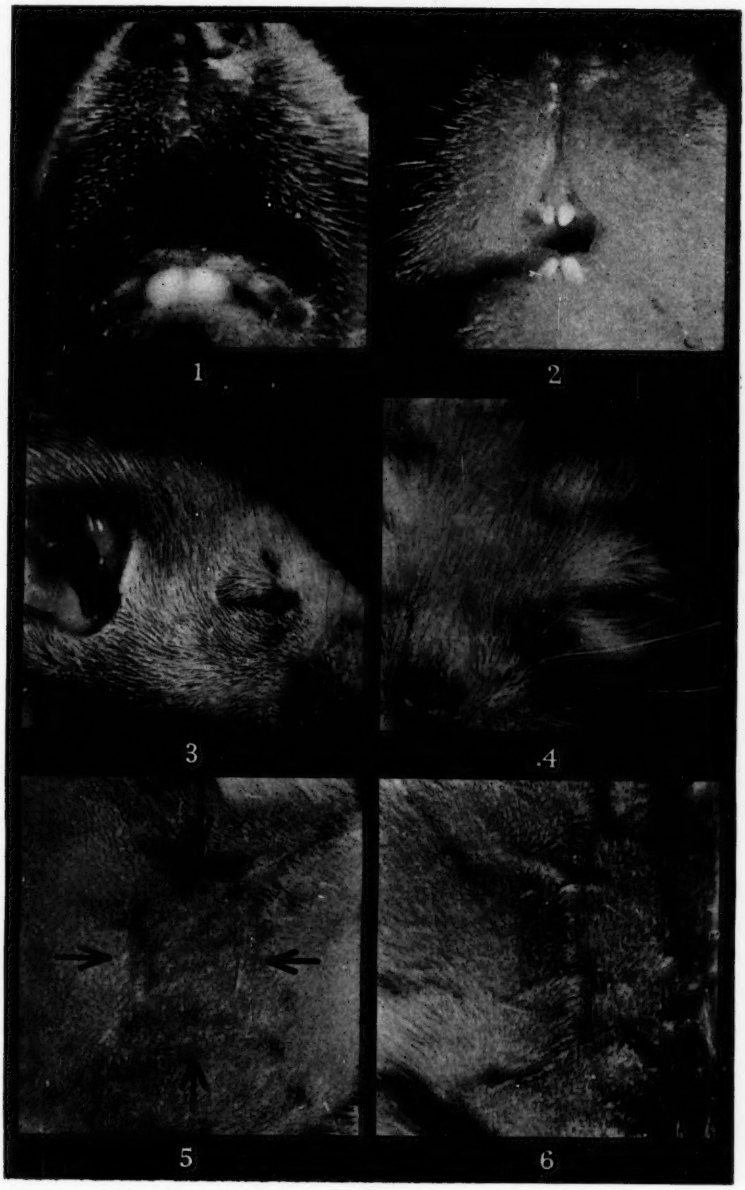
EXPLANATION OF PLATES.

FIG. 1.—Mouth of 7-day-old untreated rat showing teeth not yet erupted.
FIG. 2.—Mouth of 7-day-old rat treated from birth with cortisone, showing upper and lower incisors erupted.
FIG. 3.—Twelve-day-old untreated rat showing eye not yet opened.
FIG. 4.—Twelve-day-old rat treated with cortisone from birth showing eye opened.
FIG. 5.—Successful homograft after 12 weeks. Recipient injected with donor cells on day of birth.
FIG. 6.—Scar 12 weeks after destruction of an unsuccessful homograft.
FIG. 7, 8, 9.—First-set homograft after 2, 4 and 12 weeks respectively. (H. and E. × 135.) Minimal inflammatory reaction. Tolerance induced by injection of cells at birth. No cortisone.
FIG. 10, 11, 12.—Second-set homograft after 1, 2 and 4 weeks respectively. (H. and E. × 135.) Minimal inflammatory reaction. Tolerance induced by injection of cells at birth. No cortisone.
FIG. 13.—First-set homograft after 12 weeks, indistinguishable from an autograft. (H. and E. × 135). Tolerance induced by injection of cells at 2 weeks of age. No cortisone.
FIG. 14.—First-set homograft after 14 days. (H. and E. × 135.) Breakdown has been complete for some days. Immunity induced by injection of cells at 4 weeks of age.
FIG. 15.—Second-set homograft after 7 days showing complete breakdown. (H. and E × 135.) Same animal as in Fig. 14.



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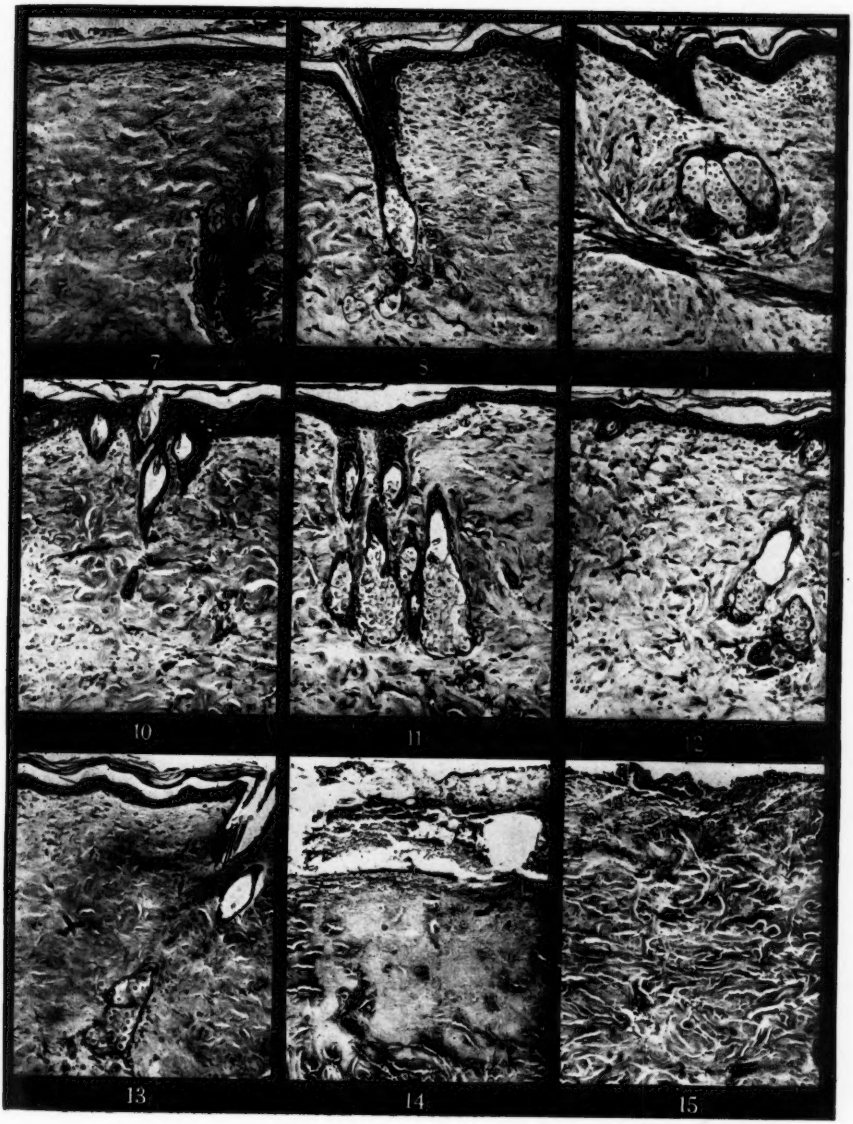
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nately these were fairly evenly distributed and the surviving animals in each of the 6 groups continued to represent 4 different combinations of donor and litter of origin of recipient.

TABLE III.—*Behaviour of First-set Skin Homografts in Rats Previously Injected with Donor Cells.*

Age of recipient when donor cells first injected.	Cortisone-treated or not.	Number of animals grafted.	Number of grafts surviving after 2 weeks.	4 weeks.	12 weeks.
New-born	No	8	8	8	8
	Yes	11	11	11	11
2 weeks	No	12	3	2	2
	Yes	15	6	4	4
4 ,,	No	8	0	0	0
	Yes	8	0	0	0

TABLE IV.—*Behaviour of Second-set Skin Homografts in Rats Previously Injected with Donor Cells.*

Age of recipient when donor cells injected.	Cortisone-treated or not.	Survival of first-set graft for 12 weeks or not.	Number of animals receiving second-set grafts.	Number of second-set grafts surviving after 1 week.	2 weeks.	4 weeks.
New-born	No	No	0	0	0	0
		Yes	7*	7	6	6
	Yes	No	0	0	0	0
		Yes	10*	10	10	10
2 weeks	No	No	3	2	0	0
		Yes	1*	1	0	0
	Yes	No	5	2	0	0
		Yes	4	4	3	3
4 ,,	No	No	4	0	0	0
		Yes	0	0	0	0
	Yes	No	4	0	0	0
		Yes	0	0	0	0

* One animal which failed to survive the operation has been excluded.

In the nineteen animals injected with foreign cells on the day of birth, all the first-set grafts survived throughout the whole period of observation (6 months), and were macroscopically indistinguishable from autografts (Fig. 5). Microscopically (Fig. 7, 8, 9) there was usually evidence of a mild inflammatory reaction characterised by some accumulation of round cells, especially in the 2-week biopsy specimens, and by some newly-formed connective tissue in the graft bed in the 4-week specimens; by 12 weeks, however, little or no trace of this reaction remained.

The second-set grafts in these animals behaved somewhat less uniformly. Nine grafts, 7 in animals which had received cortisone in infancy and 2 in animals which had not received cortisone, resembled the first-set grafts, remaining unchanged in size and evoking only a mild and temporary inflammatory reaction (Fig. 10, 11, 12). Seven grafts, 3 in animals which had received cortisone and 4 in animals which had not, evoked a rather more marked reaction, predominantly fibroblastic in character, and gradually contracted until, after 4 weeks, they were reduced to between a half and a quarter of their original area. One graft, in an animal which had not received cortisone, was destroyed and cast off in less than two weeks; the first-set graft, however, showed no change.

In 18 of the 27 animals which were injected with donor cells two weeks after

birth, breakdown of the first-set graft was just complete at the time of the first biopsy, and in 3 animals breakdown occurred between 2 and 4 weeks after grafting. In 6 animals, 4 of which had received cortisone in infancy and 2 of which had not, the grafts evoked only a mild reaction and survived throughout the whole period of observation (Fig. 13).

Fourteen animals, including 5* of the 6 with surviving first-set grafts, received second-set grafts. In 3, all animals which had received cortisone in infancy and showed surviving first-set grafts, the second-set grafts evoked a fairly marked reaction but survived throughout the period of observation. In the remaining animals the second-set grafts were destroyed within two weeks.

In all the animals which were injected with donor cells at the age of four weeks, irrespective of whether they received cortisone or not, the breakdown of both first- and second-set grafts was complete at the time of the first inspection, 2 weeks and 1 week after grafting respectively (Fig. 14, 15). The histological findings suggested that breakdown of the first-set grafts had actually occurred several days before they were inspected.

It has been shown previously (Woodruff and Simpson, 1955), using the same two strains of rats as in the present experiment, that 2 × 2 cm. split-skin homografts from hooded donors to *untreated* Wistar recipients survive nearly always for 13 or 14 days, and second-set grafts, transplanted 3 to 10 weeks after the first-set, survive 4 or 5 days. No first-set grafts survived longer than 18 days, nor were any second-set grafts alive when inspected 7 days after grafting.

The following conclusions may therefore be drawn from the present results.

Recipients injected with homologous cells (in the dose stated) on the day of birth are, when grown up, completely, or almost completely, tolerant of skin homografts from the same donor.

Injection at 2 weeks of age confers a high degree of tolerance in a few recipients and a lesser degree of tolerance in others ; in the majority of animals, however, it does not cause either tolerance or immunity.

Injection at 4 weeks of age induces a state of immunity to skin grafts from the same donor.

The probability that injection of homologous cells to new-born, 2-week-old and 4-week-old rats respectively, will result in tolerance to donor skin is not significantly altered by administration of cortisone in the dosage, and for the period, stated. On the other hand, when tolerance does develop it tends to be a little more complete in cortisone-treated animals.

A second-set graft, made to an animal with a long-established first-set graft from the same donor, may sometimes break down while the first-set graft remains unchanged.

DISCUSSION.

The present findings, together with those reported previously (Woodruff and Simpson, 1954), confirm the conclusion of Billingham *et al.* (1953) that animals injected at a sufficiently early stage of development with foreign homologous cells will later tolerate homografts from the same donor. They show in addition that in the rat, as distinct from the mouse, the period during which tolerance is conferred extends a little beyond the phase of intra-uterine life. Any hypothesis designed to account for the phenomenon of induction of tolerance must take cognisance of this

* The sixth animal unfortunately died during the second grafting operation.

fact, and of the further finding that treatment with cortisone for a period immediately following the injection of foreign cells does not decisively modify the effect, or lack of effect, of this injection.

In each age group the dose of cortisone employed was sufficient to cause clear-cut changes in development, and would seem, in the light of the observations of Parmer, Katonah and Angrist (1951), and Wells and Kendall (1940), to have been about half the lethal dose ; it is hardly likely therefore that the negative findings with respect to the induction of tolerance were due to inadequate dosage. The effect of cortisone treatment in accelerating eruption of the teeth and opening of the eyes of young rats has been reported previously by Parmer *et al.* (1951) but would seem to merit more detailed investigation.

The observation that a first-set graft which has been established for four months may continue to survive while a second-set graft from the same donor is destroyed provides further evidence in support of the writer's hypothesis of the critical period (Woodruff, 1952 ; 1954), according to which homotransplants become progressively less vulnerable as time goes on, and after a certain critical period are capable of surviving in the face of a degree of immunity in the host which they would not have withstood at an earlier stage in their life history. This hypothesis was introduced to account for some observations on the behaviour of grafts in the anterior chamber of the eye ; the findings now under consideration suggest, however, that it may have a wider application.

SUMMARY.

Rats injected with homologous cells on the day of birth are, when they grow up, almost completely tolerant of skin homografts from the same donor. Injection at 2 weeks of age confers a high degree of tolerance in a few recipients and a lesser degree of tolerance in others ; usually, however, it causes neither tolerance nor immunity. Injection at 4 weeks of age induces a state of immunity to skin grafts from the same donor.

The probability that injection of homologous cells to new-born, 2-week-old, and 4-week-old rats respectively, will cause tolerance to donor skin is not altered by administration of cortisone, although when tolerance does develop it tends to be a little more complete in cortisone-treated animals. A second graft, made to an animal with a long established graft from the same donor, may sometimes break down while the first graft remains unchanged. The significance of these observations is discussed.

We are indebted to Mr. C. Waldren and Mr. F. Aitken for technical assistance, and to the University of Otago Medical School Photographic Unit for the illustrations. Part of the cost of the investigations was defrayed by a grant from the New Zealand Medical Research Council.

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THE INFLUENCE OF *AEROBACTER* LEVAN ON THE PERMEABILITY OF THE BLOOD VESSELS OF THE SKIN: STUDIES WITH ANTIBODY GLOBULINS AND TRYPAN BLUE.

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LARGE molecules, circulating in the blood plasma, might enter into the interstices of the capillary endothelium, and alter its sieving characteristics. The supposition that circulating polysaccharides have this effect and thus limit the colloid permeability of the endothelium in an area of inflammation is in accord with the finding that native levan of *Aerobacter levanicum* suppresses the acute inflammatory response of skin to injected *Staphylococcus pyogenes* (Shilo, Wolman and Hestrin, 1954; Shilo, Wolman, Wolman, Davies and Hestrin, unpublished results) and lowers the resistance of the mouse to intraperitoneal infection with *Salmonella typhi* (Hestrin, Shilo, Feingold and Wolman, 1954).

Chambers and Zweifach (1940) and Danielli (1940) showed that oedema of organ and tissue preparations is decreased by the presence of colloids in the perfusion fluid, and it was suggested that this effect is in part due to an endothelium-sealing activity (ESA) on the part of the colloids (Chambers and Zweifach, 1947; Danielli and Stock, 1944; Bickel and Dieckhoff, 1954). More recently, Day (1952) has shown that fluid flow through connective tissue is decreased by the impaction of molecules of dextrans and related high polymers into the tissue.

In view of the many possible uses of an antiphlogistic agent in experimental biological research and in therapy, further investigation of the action of levan on the inflammatory process appeared desirable. In this communication evidence is presented that the permeability of the capillary endothelium of the skin towards serum antibody proteins and trypan blue is lowered by the systemic administration of levan.

MATERIALS AND METHODS.

Preparation of levan.

Aerobacter levanicum (Hestrin, Avineri-Shapiro and Aschner, 1943) was grown in a liquid medium containing "Bactopeptone" 1 per cent, NaCl 0·5 per cent, Na_2HPO_4 0·25 per cent, meat extract (Bovril) 0·3 per cent and sucrose 6 per cent for three days at 29°. The cells were spun down by rapid centrifugation and two vol. of ethanol were added with stirring to the supernatant fluid. The precipitated gum was purified by repeated solution in water and precipitation by ethanol, and was finally converted into a non-hygroscopic white powder by persistent trituration under renewed portions of absolute methanol. The material was dissolved at 3 per cent concentration in water at pH 6–7 and heated to 80° for 15 min. A gelatinous impurity which was coagulated by this procedure was removed, and the heat treatment repeated until no further precipitate was produced by heating. The levan in

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solution was again precipitated by methanol and dried with petroleum ether. The ketose equivalent (determined colorimetrically by the method of Roe, Epstein and Goldstein (1949) of the dry levan (after correction for ash) showed that the final product was over 98 per cent pure.

Administration of levan.

Levan was injected slowly as a 3 per cent solution in physiological saline roughly equiviscous with blood. To reduce incidental infection solutions were heated at 80° for 15 min. before injection. Unless otherwise indicated, levan was injected intravenously.

Toxicity tests.

Preparations of levan were examined for toxicity by intravenous and intraperitoneal injection in groups of inbred laboratory mice, hamsters and guinea-pigs, in doses of up to 500 mg. per kg. body wt. Mongrel rabbits were given similar doses intravenously. Out of six batches of levan tested, four proved non-toxic and were used in the present experiments.

Levan injected intraperitoneally into groups of ten mice on five successive days (500 mg./kg. per injection), caused no evident symptoms of illness or loss of weight, but at autopsy these animals showed some enlargement of the spleen.

Toxins and antitoxins.

Staphylococcus α-toxin (freeze-dried) and staphylococcus antitoxin were refined preparations kindly supplied by Dr. Sterne of the Wellcome Research Laboratories. The 50 per cent haemolysis dose of toxin in a test system containing toxin and 2 per cent rabbit erythrocytes in veronal buffer (Mayer, Osler, Bier and Heidelberger, 1946) was 1–3 μg./ml. One μg. of toxin was neutralised by approximately 10^{-5} ml. of antitoxin solution.

Diphtheria toxin solution was a purified concentrate (3400 Lf/ml.) kindly supplied by Dr. C. G. Pope. Diphtheria antitoxin was a solution of enzyme-refined globulins (5000 i.u./ml.) marketed by Burroughs, Wellcome & Co.

Skin tests.

The hair was clipped and the exposed skin further depilated with abarium sulphide paste. Intradermal injections of toxin were made in 0·1 ml. of fluid. To deliver small volumes of turpentine an "Agla" micrometer syringe was used. Trypan blue was injected intravenously as 2 ml. of a filtered 1 per cent solution of dye per kg. body wt.

RESULTS.

Influence of Levan on Extravasation of Trypan Blue.

The inhibitory effect of intravenous levan on extravasation of trypan blue at skin sites injected with turpentine is illustrated by Table I. Five groups of rabbits were given graded doses of turpentine. Series A records results on 3 groups of rabbits (4 per group) which received levan or saline intravenously followed by

TABLE I.—*Effect of Levan on Extravasation of Trypan Blue in Areas of Skin Injury.*

Series.	Turpentine dose (ml.).	Levan (mg.). 0	200	100*	50	25	15
A	0·005	+	−	−	−	±	+
	0·010	+	−				
	0·100	+	+				
B	0·005	+	−				
	0·010	+	−				

* Tests at levan doses of 100 mg./kg. and less were performed each on one rabbit.

Results were read as: + = pronounced local blueing evident within 5 min. after injection of dye. ± = local blueing reaction negative after 15 min. and slight after 60 min. − = no visible local blueing after 15 and 60 min.

turpentine intradermally after about 10 min., and after a further 10 min. by trypan blue. In Series B 2 groups of 2 rabbits each were given turpentine intradermally and 3 hr. afterwards levan or saline intravenously, trypan blue being given intravenously about 10 min. afterwards. When peptone (10 mg. "Difco" proteose peptone in a vol. of 0·1 ml.) was used to produce the skin injury, the effect of levan was similar. Levan prevented the visible escape of dye at sites challenged by 0·005 ml. turpentine but failed to control response at sites challenged with 0·1 ml. of turpentine. The levan was equally effective whether given up to 1 hr. prior to challenge (series A of Table I) or 3 hr. after the challenge (series B). This suggests that levan is able to seal already injured endothelium, *i.e.*, it does not necessarily act only as a prophylactic.

The effect of levan on dye extravasation was further studied in the Syrian hamster. Animals (5 per group) received levan intravenously (500 mg./kg.) about 30 min. before the dye, controls receiving saline instead of levan. Blueing of the extremities (paws and snout) appeared only after a relative delay in the levanised group (10–60 min. as against 5–15 min. in controls). Extravasation of dye, under pentobarbitone anaesthesia, at sites of incision in the scrotum and exposed tunica vaginalis, and at sites of mechanical injury in the cheek pouch, was much slower in the levanised animals than in the control group.

Influence of Levan on Dermonecrosis.

Staphylococcus α-toxin.

Experiments testing the effect of intravenous levan on the dermonecrotic response to staphylococcus α-toxin in rabbits passively immunised with antitoxin are set forth in Table II. Rabbits received levan intravenously 60 min. before intradermal challenge with graded dilutions of staphylococcus α-toxin injected

TABLE II.—*Neutralisation of Intradermal Staphylococcal α-Toxin by Intravenous Antitoxin, as Influenced by Levan.*

Group.	Levan mg./kg.	Antitoxin ml.	Dermonecrotic response to toxin (μg). 50	10	5	2·5
A	0	0	+	+	±	−
			++	+	+	+
			++	++	++	+
B	100	0	++	+	+	±
			++	+	+	+
C	0	0·01	−	−	−	−
			±	−	−	−
		0·20	±	−	−	−
			±	−	−	
D	100	0·01	++	++	+	±
			++	++	+	+
			++	+	+	−
		0·20	++	+	+	+
			++	++	+	+

Lesions were read at 48 hours as follows: ++ = solid yellow necrotic area; + = mottled red to yellow necrotic area; ± = rose coloured indurated area; − = faint pink area or no visible reaction. Each line of the fourth column represents one rabbit.

in a vol. of 0·1 ml. Antitoxin was given intravenously 10–30 min. before the toxin. In the rabbits which did not receive antitoxin the dermonecrotising action of toxin was unaffected by levan (compare groups A and B). In rabbits not given levan, antitoxin markedly reduced the damage caused by toxin. In the levanised rabbits, on the other hand, the same amounts of antitoxin were ineffective (compare groups A and D).

Antitoxin levels of serum 1 hr. after the injection of antitoxin were estimated on the basis of the haemolysin-inhibiting titre. Serum antitoxin levels were approximately the same in a levanised and non-levanised rabbit. Moreover, added levan at a concentration of 1 per cent did not affect the amount of toxin producing lysis of red cells *in vitro*. The minimum dermonecrotising dose of toxin was not altered by presence of 3 per cent levan in the injected toxin solution in a normal or in a passively immunised rabbit. The centrifugation at high speed (15,000 r.p.m. for 60 min.) of plasma from a levanised, passively immunised rabbit, resulted in the partial deposition of the levan without any detectable decrease in antibody content of the supernatant plasma or concentration of antibody in the deposited gel. Thus it may be concluded that levan does not interfere directly with the interaction of toxin and antitoxin, that levan does not appreciably bind the antitoxin in plasma and, finally, that it does not act directly on the skin cell and sensitise it to toxin.

These findings all accord with the explanation that levan enhances the dermonecrotic response to toxin in the passively immunised animal by inhibiting the transudation of antitoxin from the capillary bed into the area of inflammation. Further evidence in support of this thesis may be derived from the following experiments.

Diphtheria toxin.

The effect of levan on local escape of antitoxin from the vascular bed to an area of toxin challenge was studied in the passively immunised guinea-pig injected intradermally with diphtheria toxin. The guinea-pig is a particularly suitable subject for this test as it lacks natural immunity to diphtheria. Furthermore, the action of diphtheria toxin on the skin cells of the guinea-pig cannot be reversed by antitoxin given after the first few hours (Miles, 1953). Thus, even though the visible signs of necrotic response to this toxin develop only after a lapse of 48 hr., they must reflect a situation which prevailed during a defined early interval in the experiment which is summarised in Table III. Guinea-pigs were given levan or

TABLE III.—*Neutralisation of Intradermal Diphtheria Toxin by Circulating Antitoxin as Influenced by Levan.*

Each value tabulated below represents the results of a titration in the skin of one animal.

Levan dose mg./kg.	Enhancement.	Minimum dose of toxin eliciting haemorrhagic dermonecrosis.
0	—	>200, 200, 200, 200, 200, 200*
12	None	200, 200
25	Slight	200, 20
50	Definite	20, 20
100	,,	20, 2 (died)
150	Marked	2, 2, 2*
500	,,	2, 2

* 250 i.u. of antitoxin only.

saline intraperitoneally and diphtheria antitoxin (500 i.u.) was given intraperitoneally about 30 min. later. After a further 30 min. 0·2, 2, 20 and 200 Lf $\times$ 10^{-3} of toxin respectively in vol. of 0·1 ml. were injected intradermally. Lesions reached full development after two days and enhancement of the reaction by levan was assessed on the basis of area and severity of dermonecrosis. This varied from a pink spot to mottled reddish or solid red haemorrhagic area to solid black haemorrhagic area of necrosis.

Visible dermonecrotic response to diphtheria toxin was evoked in the passively immunised guinea-pig by a much smaller dose of diphtheria toxin in the presence of levan in the circulation, than in its absence. Moreover, the area of the dermonecrotic damage was much greater in the levanised animals than in the controls (see Plate). Even though the number of animals observed at any dose level was of necessity small, the results suffice to show a positive relationship between levan dose and observed effect. Under the conditions of this experiment the minimum reactive dose of levan was about 25–50 mg./kg., maximum effect being obtained with about 150 mg./kg. A similar dependence of effectivity on levan dosage had been apparent in the earlier experiments in which trypan blue served as indicator of colloid transudation.

Influence of Structure of Polymer and Degree of Polymerisation on ESA.

The activities of dextrans, polyvinylpyrrolidones and levans of different degrees of polymerisation in the diphtheria toxin-antitoxin test system are examined in the experiments shown in Table IV. Dextrans of defined molecular weight were kindly provided by Dr. Homer E. Stavely, Commercial Solvents Corp., U.S.A., and polyvinylpyrrolidones by Dr. W. Wither, General Aniline and Film Corp., U.S.A. Dr. Feingold of this laboratory prepared the levan fraction sub A VI 73 by alcohol fractionation of a partial hydrolysate of native levan. It is pertinent from the standpoint of the use of polymers as plasma volume expanders to note that in every case the polymer fractions of molecular weight below 10^5 showed no ESA in the conditions of the test. On the other hand the fractions of both dextran and polyvinylpyrrolidone having molecular weights in the range 10^5–10^6 all show some activity although their effect is not as uniform, nor as marked, as that of the native levan. *Aerobacter* levan has a very large molecular weight and a highly branched structure (Feingold, 1955 ; *cf.* also Bell and Dedonder, 1954), and it seems likely that these features are pertinent to the relatively high ESA of this polymer.

EXPLANATION OF PLATE.

Guinea-pigs (250 g.) were given 250 units of diphtheria antitoxin preceded by either saline (Fig. 1) or 3 per cent levan (Fig. 2) intraperitoneally. Thirty minutes later, the following doses (Lf $\times$ 10^{-3}) of diphtheria toxin were injected intradermally into the abdominal quadrants :

100	2
4	20

Photographs were taken after 2 days. The animal receiving levan shows skin necrosis at $\frac{1}{50}$th of the minimal reacting dose in the control animal.

(Photographs by Mr. H. D. Reuveni.)

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TABLE IV.—*Effect of Type and Molecular Weight of Polymer on Dermonecrotic Reaction Elicited by Diphtheria Toxin in Passively Immunised Guinea-pigs.*

Polymer (200 mg./kg.) was injected intraperitoneally, antitoxin (250 i.u.) intraperitoneally at 30 min. and the test dilutions of diphtheria toxin (2, 4, 20 and 100 Lf × 10^{-3}) at 60 min. intradermally. Each value listed in the fourth column represents a different animal.

Polymer.			Response.	
Type.	Designation.	Order of molecular weight.	Minimum dose of toxin producing dermonecrosis.	Enhancement.
None	—	—	100, 100, 100	—
Levan	Native	10^7–10^8	2, 2, 2, 4, 4	Marked.
	Sub A VI 73	24,000	100, 100	Slight.
Dextran	Native (CSC)	$\geqslant 10^6$	2 (died), 2 (died), 20, 100	Variable.
	NRC8 (CSC)	410,000	2 (died), 20, 20	Moderate.
	NRC7 (CSC)	250,000	2, 20	,,
	Clinical (CSC)	60,000	100, 100, 100	None.
	Macrodex (Pharmacia)	60,000	100, 100, 100	,,
Polyvinylpyrrolidone	K 90	350,000	4, 4, 4, 100	Moderate.
	Plasdone C	45,000	20, 100, 100, 100	None.

Since levans and dextrans vary in chemical structure and molecular size with biological source and the environmental conditions prevailing during their synthesis, it is to be expected that variations will occur between the ESA of different preparations of these polysaccharides. In view of the recent trend towards the use of dextrans for transfusion with molecular weights above 10^5 (Boyd, Fletcher and Ratcliffe, 1953; Martin, 1955), detailed examination of the effect of molecular size and shape of a polymer on ESA may be of importance.

TABLE V.—*Summary of Observations on Blood Pressure and Flow in Levanised Animals.*

Levan dosage: 100 mg./kg. for rabbits and hamsters, 500 mg./kg. for mice.

	Animal.				
		Number.			
	Species.	Levan.	Controls.	Method.	Findings in levanised animals.
Central blood pressure	Rabbit	2	0	Carotid canula	Normal after brief drop (1).
Rectal temperature	,,	4	4	Mercury thermometer	No change.
Skin colour	,,	4	4	Inspection	,, ,,
Skin temperature	,,	2	2	Constantin-copper thermocouple	Normal fluctuations.
Ear skin reactivity	,,	1	1	Superficial stimuli (2)	Normal temperature rise.
Flushing response	Mouse	6	6	Hind limb 45°, 30 sec.	Normal reaction (3).
Pattern of vascular bed	Hamster	3	0	Microscopy of living cheek pouch preparation (4)	No change.

(1) Following injection of levan, pressure fell rapidly from 110 mm. Hg to 70 mm. and reverted to normal steady level after about 8 min. Pentobarbitone anaesthesia.
(2) Scratching base of ear with finger nail; rubbing of ear tip with toluene-soaked swab.
(3) Flushing of limb normal as to speed, extent and duration.
(4) Fulton, Jackson and Luntz (1947).

Observations on Blood Pressure and Flow.

It has been demonstrated that animals in shock show reduced egress of serum colloids and cells from the circulation to areas of skin injury (Miles and Niven, 1950). It was therefore relevant to ascertain whether the effects of levan described above were due to the production of shock in the experimental animals. Observations on this point are summarised briefly in Table V, from which it is evident that the animals given levan were not shocked. Furthermore, no evidence has been found that levan has vasodilator or vasopressor activity, nor did levan change noticeably the ability of the vascular bed to respond in the typical manner to simple mechanical stimuli.

SUMMARY.

Native levan of *Aerobacter levanicum* limited the capacity of the blood vessels of the skin to respond to local injury with increased permeability to trypan blue.

The severity of the dermonecrotic response to injection of staphylococcus α-toxin and diphtheria toxin in passively immunised animals was enhanced, and the minimum dermonecrotising dose of toxin lowered many fold, by presence of native levan in the circulation at the time of challenge.

Polymer preparations of levan, dextran, and polyvinylpyrrolidone of molecular weight lower than 10^5 were inactive in the above system. Higher polymer fractions of dextran and polyvinylpyrrolidone showed endothelial sealing activity inferior to that of native levan.

It is suggested that the permeability of the vascular bed of the skin towards antibody globulins and other similarly large molecules is lowered by the entry of levan molecules into the lattice of the capillary endothelium.

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A MICROSCOPICAL STUDY OF THE REPRODUCTION OF PSITTACOSIS VIRUS.

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Soon after Bedson, Western and Simpson (1930) first demonstrated that psittacosis is caused by a filterable virus, a number of workers described the intracytoplasmic inclusions and " plaques " that are so characteristic of the tissues of animals subjected to this infection (Bedson and Western, 1930 ; Coles, 1930 ; Levinthal, 1930 ; Lillie, 1930).

Bedson and Bland (1932; 1934) made detailed morphological studies of the virus as it is seen in stained and fixed preparations of the spleens of infected mice. Bland and Canti (1935) recorded the changes occurring in tissue cultures of infected chick embryo cells by means of motion photo-micrography under dark ground illumination. From these observations it was concluded that psittacosis virus undergoes a complex developmental cycle in which the various changes are linked both in time and morphological sequence.

There is little or no evidence to suggest that this developmental cycle is characterised by an eclipse phase analogous to that postulated for the bacterial viruses. Indeed the results of a recent re-investigation of this question by Bedson and Gostling (1954) based on the microscopy of stained smears and on serological tests for the various forms of the virus together with animal infectivity experiments, give strong support to the opinion that the virus reproduces by a process of binary fission.

The simple hypothesis of binary fission does not however explain the part played in the reproductive process by the " plaques " of the earlier workers. The work of Bland and Canti indicates that these inclusions are in fact colonies of virus embedded in a matrix and enclosed, at least for a time, by a limiting membrane. The limitations of the resolving power of the optical microscope and the disadvantages inherent in dark ground illumination studies precluded the accurate measurement of the range of particle size by these workers and denied to them the observation of the fine details of the intracellular changes. It was therefore decided to examine again the reproductive cycle of psittacosis virus using the greater resolving power of the electron microscope and the improved definition of the structural detail of unstained cells made available by phase contrast microscopy.

MATERIALS AND METHODS.

Strain of virus.—The M.O.H. 154 strain, obtained through the kindness of Professor S. P. Bedson, was used throughout. This strain was adapted to the yolk-sac of the chick embryo and had been passaged in this situation 70 times in the author's laboratory before

this investigation began. Its virulence was such that over 90 per cent of the embryos were killed regularly by 0·1 ml. of a 1/10 dilution of infected yolk sac emulsion 72 hr. after inoculation by the method of Bedson, Barwell, King and Bishop (1949).

The strain was also adapted to the mouse by intraperitoneal inoculation and after 15 serial passages 0·5 ml. of a 5 per cent suspension of infected spleen killed the animals in 48–60 hr.

Strain of mice.—Albino mice from the same stock, 4–8 weeks old, were used throughout.

General plan.

At timed intervals photographic records were made of the changing morphology of the virus during its reproduction. The results of a series of observations, recorded by phase contrast photomicrography, on the cells of the spleen of the infected mouse, were compared with a series of electron micrographs of the various forms of the virus obtained at intervals from the yolk-sacs of infected eggs.

Microscopical examinations.

Optical microscopy.—Impression smears were stained by Bedson's modification of Castaneda's method after fixation with 0·1 N-HCl, by Machiavello's stain, and by Giemsa after alcohol fixation.

Phase contrast microscopy.—Wet preparations of fresh unfixed splenic cells were made from infected mice and from controls which had been inoculated with sterile nutrient broth. To prepare the cell suspensions used in the wet films the spleen of a mouse was removed immediately after the animal's death and was washed in three changes of buffered saline; after fine mincing it was suspended in 1·0 ml. of 5 per cent horse serum in physiological saline and agitated by repeated suction in a wide-bored Pasteur pipette. After allowing the coarse fragments to settle, the supernatant which was rich in detached cells was used to make wet films. Duplicate dry films were prepared for staining at the same time.

Electron microscopy.—Sufficient amounts of the younger forms of the virus could not be obtained from the spleens of infected mice. Pooled yolk-sacs of infected eggs were therefore used as the source of the virus for this part of the work which was controlled by the use of impression smears stained by Castaneda's method.

Three pools, each of at least eight infected yolk-sacs, were collected as follows. The first pool was obtained from infected eggs killed by refrigeration 12 or 18 hr. before the expected death of the embryos; only those yolk sacs in which the large form of the virus predominated in stained impression smears were included. Approximately 20 per cent of the eggs inoculated fulfilled this requirement. The second pool was prepared from eggs which it was anticipated would have died within 6 hr. and in which the movements of the embryo were very sluggish when examined by trans-illumination. The third pool was composed of the yolk-sacs of embryos recently killed by the infection and in which there was a satisfactory content of elementary bodies.

Control preparations were made from the pooled yolk-sacs of uninoculated eggs from each batch used for virus cultivation.

Purification and concentration.

Yolk-sacs were rinsed once in buffered saline and were then broken up by gentle shaking with glass beads. By this method coarse particles of yolk and tissue débris were not fragmented and they could be removed easily by centrifugation. This suspension was then clarified by centrifuging for 5 min. at 1000 r.p.m. The supernatant was then removed and spun in an angle centrifuge for 3 hr. at 4500 r.p.m. This cycle of differential centrifugation was repeated twice and the final deposit was re-suspended in buffered saline. A satisfactory product was only faintly turbid and when a film was stained by Castaneda's stain showed a reasonable virus content.

A drop of the final suspension was placed on a glass slide and fixed for 5 min. in osmic acid vapour. A loopful was then transferred to a collodion membrane on an electron microscope grid and dried over calcium chloride. Dissolved salts were removed by rinsing the grid in distilled water and after drying once more the preparation was shadowed with gold palladium alloy at an angle of 15°. The preparations were examined in an E.M.3 Metropolitan Vickers electron microscope using an accelerating voltage of 75 kV.

RESULTS.

Although Bedson and his colleagues have studied extensively the morphology of psittacosis virus as it is seen in stained impression smears taken from the spleens of infected mice, there is no record of the sequence of events as it occurs in the living cells of this animal. For this reason, and to confirm that the strain of virus used for electron microscopical work reproduced in the classical manner, the morphology of fresh unstained and unfixed virus was recorded photographically under the phase contrast microscope and studied in replicate Giemsa-stained smears.

Phase contrast studies.

Up to 16 hr. after infection no general changes in the host cells could be detected, the nuclei, nucleoli and mitochondria were normal and no virus particles could be recognised with certainty. From 16 hr. onwards it was possible to find within the cytoplasm of some of the mononuclear cells spherical bodies 1·0–2·5 μ diameter; sometimes compact clumps of between 4 and 8 of these bodies were present but more usually they were dispersed in the cytoplasm (Fig. 1).

When infection had continued for 20–24 hr. the virus particles had increased considerably in number and had assumed varying sizes ranging between 0·5 and 1·5 μ in diameter; they were for the most part scattered unevenly throughout the whole cytoplasm of the cell (Fig. 2). In about half of the infected cells, however, the particles were massed closely together to form a colony of the virus which appeared to be surrounded by a well defined envelope. Within the colony, constrictions and indentations in the outlines of the larger particles strongly suggested that active division was proceeding (Fig. 3).

From this time onwards the colonies increased in size and although the particles became smaller it was always possible to distinguish considerable variation in their size. On infrequent occasions two or even three colonies could be seen within the cytoplasm of a single host cell. Between 24 and 36 hr. after infection the virus particles, by this time 0·5 μ or less in diameter, were undergoing rapid Brownian movement within the colony envelope. Sometimes colonies of this type could be seen extracellularly both in fresh preparations under the phase contrast microscope and in stained smears; it was presumed that they had escaped from the host cells as a result of trauma during preparation.

The matrix of colonies 24 hr. old was rather opaque and the virus particles were motionless, while in colonies 36 hr. old and of greater size it became progressively more transparent until the stage of Brownian movement was reached. It thus appeared that concurrently with virus multiplication alterations were occurring in the viscosity and refractive index of the matrix. It was however always possible to find localised collections of virus particles in which no trace of a membrane or matrix could be detected and it was felt that this structure was not a constant feature of the reproductive process. Studies of stained films showed that the matrix does not react with Lugol's iodine and that it takes a faint pink colour with Giemsa.

When infection had continued for 48 hr. or longer no sign remained of any envelope containing the elementary bodies. Brownian movement had ceased. The cytoplasm of the host cells was filled with large numbers of elementary bodies all more or less uniform in size (Fig. 4). Meanwhile the host cells themselves

manifested evidence of damage, the mitochondria were not visible, the nucleus became increasingly transparent, and nucleoli could no longer be distinguished. Vacuolation of the cytoplasm was often prominent. Finally the cell membrane, which was visible throughout, ruptured and the elementary bodies were set free. These findings are in close accord with those of Bland and Canti and also with the observations of Salaman (1953) who described the reproductive cycle of the virus as he saw it in whole mounts of amniotic membranes of infected eggs by means of phase contrast and ultraviolet light microscopy.

Electron microscope studies.

Since yolk-sac cultures alone provided a sufficient yield of the larger virus particles they were used as the source of material despite the disadvantage that the morphological sequence of changes in the growing virus is less clearly demonstrable in the egg than in the tissues of the mouse.

However between 36 and 54 hr. after inoculation some 20 per cent of the yolk-sacs were found to contain considerable numbers of the large form of the virus as judged from impression smears stained by Castaneda's method (Fig. 5). By using sufficiently large batches of eggs it was therefore possible to obtain enough material for purification. Similarly between 54 and 68 hr. after inoculation, virus particles of a range of intermediate sizes were found to predominate especially at a time when the embryo was obviously affected by the virus. When the yolk-sacs of embryos dying some 72 hr. after inoculation were examined the virus was present in the form of elementary bodies of more or less uniform size (Fig. 6).

In every specimen examined varying numbers of elementary bodies were found. These appeared as rounded particles and measurements of 50 show that they vary in diameter between 400 and 500 mμ, the average being 450 mμ. Their appearance is that of spheres which have collapsed as a result of drying, leaving a heaped up central mass of protoplasm and a thin flattened peripheral rim. The whole particle is covered by a rather thick membrane with a wrinkled and folded surface. At the edge of the particle two layers of the membrane are in contact and form the rim (Fig. 7).

Intermediate-sized particles were obtained from 66 hr.-old yolk-sac cultures; their diameters range from 0·50 to 0·80 μ with an average of 0·61 μ. Many of these particles closely resemble the elementary body and less than half of them show any changed structure (Fig. 9). In the larger particles the protoplasm is condensed into ill-defined multiple masses about 0·15 to 0·20 μ in diameter and the limiting membrane is not so clearly defined. Thus a suggestion of division by fission is produced.

In 15 of the large virus particles the diameter ranged between 0·85 and 1·40μ with an average of 1·12 μ. In the largest of these particles, which were obtained from 54 hr. cultures, no internal structure could be defined. They appeared as flattened discs of structureless material whose irregular edges suggest that no rigid covering membrane is present (Fig. 8). Slightly smaller particles with diameters in the region of 1·00 μ were seen to contain small spheres 0·10–0·15 μ in diameter within a very thin covering membrane. In some instances, due no doubt to trauma in preparation, these particles are ruptured. Such a particle is seen in Fig. 10 where the membrane has been broken and a great part of the contents of the particle appears to have escaped; several small rounded particles

however remain within the membrane. Similar small particles separate from recognisable virus particles are seen in Fig. 8 and 9.

Particles of this size (0·1–0·15 μ) are frequently found in normal egg fluids (Ghosh Ray and Swain, 1954) and thus their significance may be questioned. However the disposition of these particles within the membrane of large forms and the fact that they could not be detected in control preparations suggest that they may originate in the virus itself. Moreover their clear-cut edges and general appearances differ appreciably from those of the particles of normal egg fluids.

DISCUSSION.

Studies of the morphological sequence of changes in the microscopical appearances of psittacosis virus during its multiplication described by previous workers and here corroborated by phase contrast photo-micrography indicate that the growth process may be described in four overlapping stages.

In the first stage the scanty numbers of virus particles can scarcely be found microscopically, in the second a few large particles 1–2 μ in diameter are present in the cytoplasm, in the third increasing numbers of intermediate-sized particles are seen sometimes in the form of a virus colony with a matrix and a limiting membrane, and in the fourth stage large numbers of elementary bodies of uniform size are dispersed evenly throughout the host cell.

It would appear that division of the particle takes place at least twice during reproduction since the intermediate-sized particle is approximately one half the size of the large form and the elementary body is nearly half as small again.

In studies of reproduction of bacteria by binary fission the measurement of the size of the multiplying organisms and the study of their changing internal structure has yielded important results. With viruses such information can only be obtained with accuracy by electron microscopical methods. Many technical disadvantages hinder the observation of the consecutive alterations in the contents of infected cells in the electron microscope. The observations of Kurotchkin, Libby, Gagnon and Cox (1947) and of McFarlane (1949) lack a correlation of time with the sequence of the morphological changes of the virus, and the electron micrographs of Heinmets and Golub (1948) show particles which may have been distorted by the ultra-sonic vibration used in the preparatory work.

In the present electron microscopical studies three forms of the virus have been distinguished during the active phase of its multiplication. The earliest is the large form whose average diameter is 1·20 μ and which has a very thin and poorly developed covering membrane. Next follows an intermediate-sized particle with an average diameter of 0·62 μ in which multiple discrete masses of protoplasm have been formed. The final stage is characterised by mature elementary bodies of uniform size varying in diameter by only 0·05 μ from a mean of 0·45 μ; they have a thicker and stronger covering membrane and show no sign of further division by segmentation of the protoplasm.

The progressive diminution of the size of the virus particles and the alteration in their structure during rapid multiplication are analagous to the morphological changes which occur in the bacterial cell during the late lag phase and the logarithmic phase of rapid growth. The affinity of the bacterial cell for basic dyes alters during active division and so also do the staining properties of the psittacosis virus particle during the early stages of its reproduction (Bedson and Bland, 1932).

Another member of the psittacosis group, meningopneumonitis virus, has been studied by Gaylord (1954) in thin sections of the chorio-allantoic membrane at intervals after infection. This author has recognised a large initial body of the virus similarly related in time sequence to intermediate-sized particles and to elementary bodies. He has also described a single membrane covering the large particles, two or three membranes enclosing the intermediate-sized particles, and very dense central granules within the elementary bodies.

Although Gaylord has agreed that reproduction by binary fission does occur, he has introduced the concept that reproduction by endosporulation also operates and that elementary bodies represent a spore-like stage. To support this contention he has drawn attention to yeast-like buds which are to be seen in one of his electron-micrographs and to multiple masses within the large forms. The diameter of the " buds " and the intermediate-sized virus particles is of the same order. However, it is not possible to be certain whether these " buds " are truly attached to the initial bodies or whether the appearance has been produced by compression of the microtome knife or is an artefact due to the angle of cutting. " Buds " of this type were never encountered in the present work nor were they observed by earlier workers on psittacosis virus.

It may be seriously doubted whether it is biologically sound to postulate two entirely distinct means of reproduction for the same organism, especially for one so little differentiated as a virus. The step-like diminution of particle size of both the psittacosis and meningopneumonitis viruses is linked in time sequence with active multiplication. The process is most satisfactorily explained as one of simple binary fission. The small bodies within the large forms of psittacosis virus

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EXPLANATION OF PLATES.

FIGURES 1–4 are phase contrast photomicrographs of wet films of fresh unfixed cells from the spleens of mice at intervals after infection. All × 1080.

FIG. 1.—Sixteen hr. after infection. Shows eight large virus forms dispersed in the cytoplasm of the host cell.

FIG. 2.—Twenty hr. after infection. Medium-sized particles with considerable differences in diameter.

FIG. 3.—Twenty-four hr. after infection. A colony of virus particles of varying sizes embedded in a matrix. One large particle appears to have divided in four and another in two.

FIG. 4.—Forty-eight hr. after infection. The virus is entirely in the form of elementary bodies dispersed in the cytoplasm.

FIGURES 5–6.—Impression smears of yolk-sac cultures. Castaneda's stain. × 1150.

FIG. 5.—Forty-eight hr. after infection. Large forms predominate.

FIG. 6.—Seventy-two hr. after infection. A cluster of elementary bodies.

FIGURES 7–8.—Electron micrographs of purified virus suspensions from yolk-sac cultures. × 20,000.

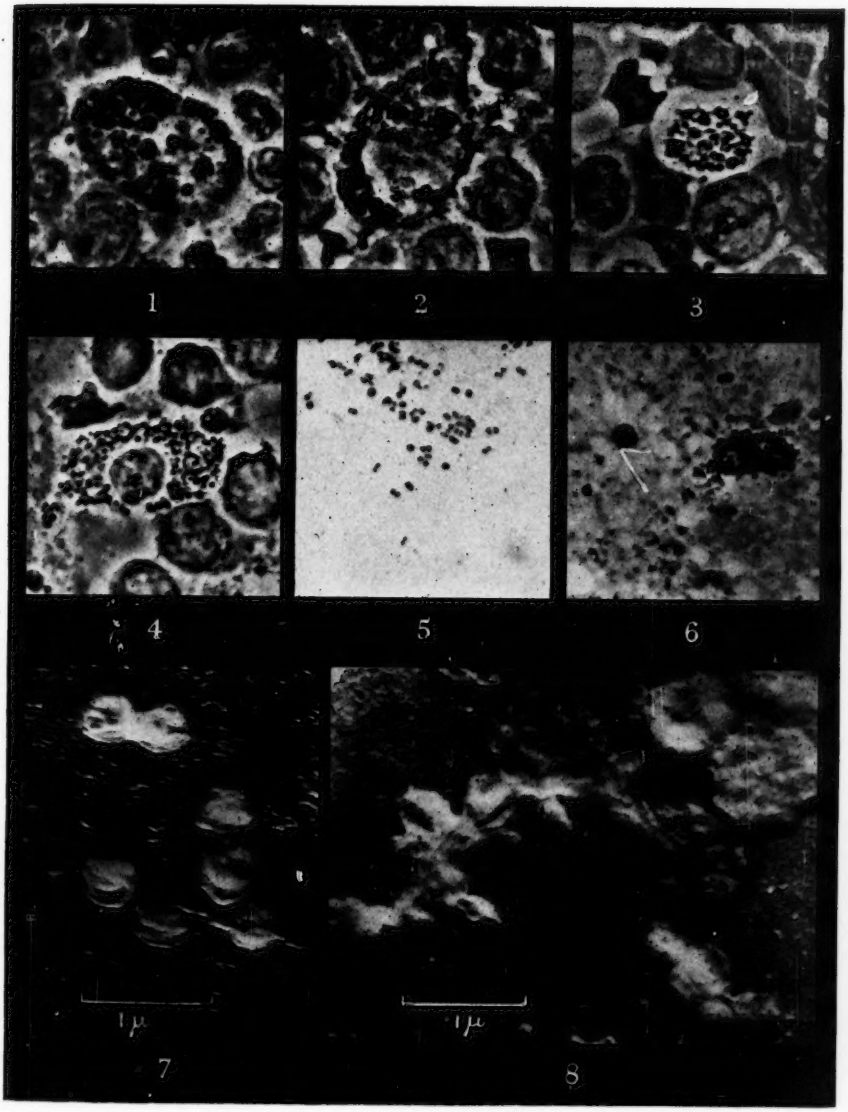
FIG. 7.—Seventy-two hr. after infection. Elementary bodies.

FIG. 8.—Fifty-four hr. after infection. Large forms together with elementary bodies. A small particle 0·10 μ in diameter is seen at the lower margin.

FIGURES 8–9.—Electron micrographs of virus suspensions purified from yolk-sac cultures. Both × 20,000.

FIG. 9.—Sixty-six hr. after infection. Elementary bodies and intermediate-sized particles, the larger of which contain several masses of protoplasm.

FIG. 10.—Fifty-four hr. after infection. Large virus particles with smaller forms. One large particle shows a broken covering membrane and appears to have been emptied of a part of its contents. Several discrete masses are seen within the membrane.


1
2
3
4
5
6
1 μ
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1 μ
8

seen in Fig. 9 and noted in the meningopneumonitis virus by Gaylord may be no more than nuclear and cytoplasmic condensations preceding division. Such masses occur in bacteria during active increase by binary fission and the observation hardly provides sufficient evidence on which to base a hypothesis of reproduction by multiple endosporulation.

If an organised colony with a well-defined architecture were an invariable and essential feature of a life cycle of psittacosis virus, there would be some difficulty in accepting the hypothesis that its reproduction is by a process of binary fission. Since, however, it is often possible to find clumps of virus particles of all sizes, without any suggestion of a matrix or covering membrane, it would seem likely that organised colonies may only occur under special circumstances. The matrix may be derived from the cytoplasm of the host cell as a reaction to the presence of the growing virus and the membrane may consist of compressed protoplasm.

An alternative explanation of the nature of the supporting structure of the colony is that it is formed by a secretory product of the virus itself. This may be true of molluscum contagiosum where van Rooyen (1939) has shown that the inclusion is covered by a membrane which, on account of its staining properties with iodine, may be composed of a carbohydrate substance. Such however is not the case with the colony of psittacosis virus of which the staining of the matrix differs only very slightly from that of the host cell.

It is of interest to note the close morphological resemblances of the elementary bodies seen in these electron micrographs to those of feline pneumonitis virus reproduced by Hamre, Rake and Rake (1947) and by Moulder (1954) where similar size variations are apparent.

SUMMARY.

The results of a phase contrast photomicrographic study of the reproduction of psittacosis virus in the spleens of mice inoculated intraperitoneally have been compared with electron micrographs of the virus obtained at timed intervals during its multiplication in the yolk-sac of the chick embryo.

Measurements of the sizes of the various forms of the virus show a decrease in the particle size with the lapse of time. Concurrently a limiting membrane is developed and the protoplasm of the virus particle is condensed into discrete masses.

It is concluded that the evidence presented supports the view that, during the active phase of its reproduction, psittacosis virus divides by binary fission.

I wish to express my thanks to Professor S. P. Bedson for his valuable criticism and to Professor T. J. Mackie for help and encouragement. I am also indebted to Mr. J. Sutherland for technical assistance.

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ACQUIRED IMMUNE TOLERANCE TO HUMAN ALBUMIN AND THE RESPONSE TO SUBSEQUENT INJECTIONS OF DIAZO HUMAN ALBUMIN.

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EVIDENCE has been accumulating that animals exposed to foreign antigens during pre-natal or early post-natal life may be unable to produce antibodies to those antigens in later life. The first indication of this behaviour was observed by Owen (1945) who found that in cattle the common placental circulation of twin foetuses could result in the presence of two antigenic types of erythrocytes in each adult twin. One of these corresponded to its own genetic constitution, the other type to that of its twin. A similar occurrence in man was later reported by Dunsford, Bowley, Hutchison, Thompson, Sanger and Race (1953). The presence in the circulation of erythrocytes of a type other than that of the individual suggests that antibodies which would lyse them were absent. Billingham, Brent and Medawar (1953; 1955) proved that the breakdown of skin grafts could be prevented or at least greatly reduced by the injection of splenic or other tissue from the donor strain into mouse foetuses. The survival of the graft was ascribed to a primary failure of the machinery of the immunological response and was named actively acquired immune tolerance.

The injection of living cells into a foetus inhibited the breakdown of a skin graft from the donor strain of mice, whereas the injection of killed cells was ineffective. With other antigens and in other animals it has been possible to inhibit antibody response with non-living antigens. Buxton (1954) inhibited antibody response in chickens by the injection of killed *Salmonella pullorum* into embryos before the 16th day of incubation, and Kerr and Robertson (1954) inhibited the antibody response of cattle by the injection of freeze-dried or acetone-dried *Trichomonas foetus* into new-born calves. Hanan and Oyama (1954) and Dixon and Maurer (1955*b*) observed the inhibition of antibody formation to bovine serum albumin in rabbits injected with that antigen during the first days after birth.

The injection of antigen into new-born animals or foetuses is, however, not the only way to inhibit the formation of demonstrable antibody. Large doses of certain antigens injected into adult animals can prevent the appearance of antibody in response to subsequent small doses. Perlzweig and Steffen (1923) reported that a single large injection of a fraction prepared from pneumococcus conferred little protection, whereas small doses of the same antigen did protect animals against a virulent culture. Felton (1949) and Felton, Kauffmann, Prescott and Ottinger (1955) showed that injections of large doses of pneumococcus

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polysaccharides into adult mice did not stimulate antibody formation and prevented subsequent small doses from doing so. This inhibition—termed immune paralysis—persists for at least 15–18 months. A similar effect has been observed in man (Felton, Cameron and Prather, 1941) and the successful use of dextran as a blood substitute may indicate that it could occur with polysaccharides other than those of pneumococcus (Maurer, 1953). Very large daily doses (0·5 g./kg. body weight) of bovine serum albumin injected into adult rabbits over a period of 6–12 weeks also prevent the appearance of demonstrable antibody (Dixon and Maurer, 1953). This effect is, however, of short duration (Dixon and Maurer, 1955*a*, *b*) and so differs from the immune paralysis caused by polysaccharides.

It appears that the inhibition of immune response brought about by the injection of antigen into foetuses, new-born or adult animals is specific for the antigen injected. Kerr and Robertson (1954) found a normal response to *Brucella abortus* in 4½ to 8 months old cattle which had been injected at birth with *T. foetus*. Dixon and Maurer (1955*b*) observed the antibody response to ^{131}I-labelled human gamma globulin of rabbits injected at birth with bovine albumin, and the antibody response to ^{131}I-labelled bovine albumin of rabbits injected at birth with human plasma protein. The antibody concentration was similar to that of animals not injected at birth or slightly lower. A reduced response to egg albumin in rabbits injected at birth with bovine serum albumin was, however, reported by Hanan and Oyama (1954).

Immune paralysis to a polysaccharide of one type did not reduce the antibody response to a polysaccharide of another type (Felton *et al.*, 1955).

Billingham *et al.* (1955) found that the specificity of acquired tolerance was complete if tolerance was complete.

The purpose of this investigation was to examine the specificity of the inhibition of immune response, by the injection of a protein at birth followed in later life by the injection of the same protein in a chemically modified form and of a completely unrelated protein.

MATERIALS AND METHODS.

Protein Antigens.

Human albumin (*H.A.*).—H.A. was prepared by ether fractionation by Dr. M. E. Mackay (Kekwick and Mackay, 1954). It was shown by diffusion in agar of excess antigen against H.A. antiserum to contain one major and two minor antigens. By electrophoresis in agar (Grabar and Williams, 1953) the two minor components migrating with a slightly lower mobility than the albumin (Fig. 1, *a*) were identified as α_1 and α_2 globulins.

Tobacco mosaic virus (*T.M.V.*).—A highly purified fraction of T.M.V. was prepared by Dr. A. Kleczkowski.

Chemically Altered Antigens.

The protein antigens were diazotised in an ice water bath. The diazonium salt of sulphanilic acid, cooled to 0°, was added drop by drop to the protein solutions and the pH was kept between 7·5–8·5 by the addition of 0·2 N-NaOH.

Diazo-human albumin (*D.H.A.*).—D.H.A. was prepared from human albumin purified by ether fractionation. The protein was coupled with the diazo compound of benzene-*p*-sulphonic acid. The sulphur content of D.H.A. was 3·14 per cent. It sedimented as a single component in the ultracentrifuge. $s_{20,w} = 4{\cdot}1$, 4·05 (0·8 per cent protein) and 4·29 (0·25 per cent protein), assuming $\bar{V}_{20} = 0{\cdot}733$. Native H.A. could not be demonstrated in D.H.A. by electrophoresis in agar (Fig. 1). An antiserum having antibodies to albumin and two α globulins reacted with two components of D.H.A. (Fig. 1, *a*), but H.A. antisera reacting with human albumin only, reacted with one component only in the D.H.A. preparations (Fig. 1, *b*).

This component had a higher mobility than H.A. D.H.A. antisera reacted with components of different mobility in H.A. and D.H.A. (Fig. 1, *c*).

Diazo-rabbit albumin (D.R.A.).—D.R.A. was prepared from rabbit albumin purified by fractionation with ether (Mackay, 1955). The protein was coupled with the diazo compound of benzene-*p*-sulphonic acid. The sulphur content of D.R.A. was 3·48 per cent. D.R.A. migrated as a single boundary in the ultracentrifuge and in paper electrophoresis. The sedimentation constant was $s_{20,w} = 4{\cdot}20$ (0·8 per cent), assuming $\bar{V}_{20} = 0{\cdot}733$.

Reagents.

All reagents used were A.R. and were dissolved in glass distilled water.

Buffer solutions.—Phosphate saline buffer (pH 7·2): 0·0525 M-Na_2HPO_4, 0·0225 M-KH_2PO_4, 0·075 M-NaCl. Phosphate saline buffer (pH 6·4): 0·0225 M-Na_2HPO_4, 0·0525 M-KH_2PO_4, 0·075 M-NaCl.

Polyvidone.—Polyvinyl pyrrolidone was obtained from May & Baker Ltd., Dagenham, as a 25 per cent w/v solution free of disinfectant. Polyvidone saline solution: 0·35 per cent polyvinyl pyrrolidone, 0·15 M-NaCl.

Sheep erythrocytes.—Defibrinated sheep blood was purchased from the Wellcome Research Laboratories. The erythrocytes were used within seven days of bleeding.

Assay of Antigens and Antibodies.

Injection and bleeding of rabbits.—Injections were made into the marginal vein of the ear or intraperitoneally with protein dissolved in 0·15 M-NaCl or subcutaneously in emulsions consisting of 3 per cent (w/v) lanoline and 45 per cent (v/v) liquid paraffin (B.P.). The rabbits were bled from the marginal vein of the ear.

Agglutinations.—Sheep erythrocytes were tanned, sensitised and agglutinated by the method of Boyden (1951) as modified by Borduas and Grabar (1953). All solutions were made up in glass-distilled water. Sheep erythrocytes were washed three times with 0·15 M-NaCl, suspended in phosphate-saline buffer (pH 7·2) to give a 2·5 per cent suspension (v/v) and were then mixed with an equal volume of tannic acid (1/20,000 w/v) in 0·15 M-NaCl; after 10 min. the tanned erythrocytes were centrifuged, washed with phosphate buffer at pH 7·2 and suspended in 0·15 M-NaCl. To 10 ml. of this suspension were added 40 ml. phosphate buffer pH 6·4 containing 2·5 mg. of antigen. After 15 min. the tanned and sensitised erythrocytes were centrifuged and washed three times with polyvidone saline solution. The concentration of this suspension was adjusted so that when 0·3 ml. was added to 2 ml. of distilled water and treated with coal gas for 5 min. the optical density of the resulting solution was 0·3 ($\lambda = 572$ mμ.) in a 5 mm. cell.

Sera were heated in stoppered tubes at 56° for 60 min.; natural sheep antibodies were absorbed with 0·1 ml./ml. of washed centrifuged sheep erythrocytes. After 1 hr. the erythrocytes were removed by centrifuging and the sera were diluted in polyvidone solutions. The final dilutions usually contained 1/2, 1/5, 1/10, 1/20, 1/50, 1/100, 1/200, 1/500, 1/1000, 1/2000, 1/5000, 1/10,000 1/20,000, 1/50,000 and 1/100,000 serum. A master series was made by careful tenfold dilutions (1/10, 1/100, etc.) and intermediate dilutions from the appropriate preceding master dilutions. To 0·5 ml. of each of the serum dilutions was added 0·1 ml. of the suspension of tanned and sensitised sheep erythrocytes.

The mixtures were left at room temperature and observations were made after the erythrocytes had settled. The agglutination titre is given as the reciprocal of the dilution of antiserum in the last tube showing a conventional degree of partial agglutination (±).

Measurement of antigen concentration by agglutination inhibition.—A standard immune serum was treated and diluted in polyvidone saline as described above. The sera to be examined for the presence of foreign antigen (H.A.) were mixed with an equal volume of polyvidone saline. One tenth ml. of the resulting solutions was added to each dilution of the standard serum (0·4 ml.), and the mixtures shaken and kept for 2 hr. at 37°. Tanned sensitised sheep erythrocytes (0·1 ml.) were then added to each tube.

Sera containing unknown quantities of foreign antigen (H.A.) were compared with samples of normal rabbit serum to which were added equal volumes of polyvidone saline solutions containing known amounts of the foreign antigen. Two control series were included: in the first 0·5 ml. of the polyvidone saline solution and in the second 0·1 ml. of normal serum mixed with 0·4 ml. polyvidone saline replaced the sera of rabbits injected with H.A. The limit of sensitivity was 2 μg. H.A./ml. serum.

Precipitin test.—Precipitin tests were set up in thin-walled glass tubes (5·0 × 0·55 cm.). The α-procedure, *i.e.*, constant antibody method, was used. Antibody amounts were kept constant and antigen amounts were varied. The volume of the final mixtures was kept constant by the addition of 0·15 M-NaCl. The stoppered tubes were immersed to ½ of the depth of the liquid column in a glass fronted water-bath at 37°. A black screen was placed behind the tubes, which were indirectly illuminated. The time taken for the first appearance of floccules, as seen through a magnifying glass, was noted. Results are stated as mg. antigen/ml. serum in the mixture flocculating first.

Methods for Examining the Composition of Antigens and Antibodies.

Diffusion in agar from opposite parallel sources (Ouchterlony, 1949; Oudin, 1952).—Washed and filtered agar was made up in 0·15 M saline (1·5 per cent agar) and poured into petri dishes. Cylindrical holes were cut in the agar with a cork borer and the bottoms of the holes covered with 2 drops of agar to form cups, which were filled with the reagents to be examined. The petri dishes were sealed with plasticine.

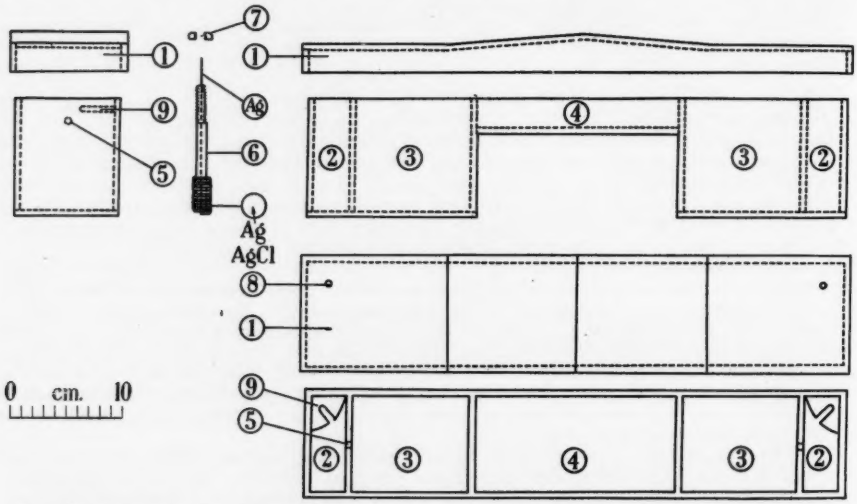


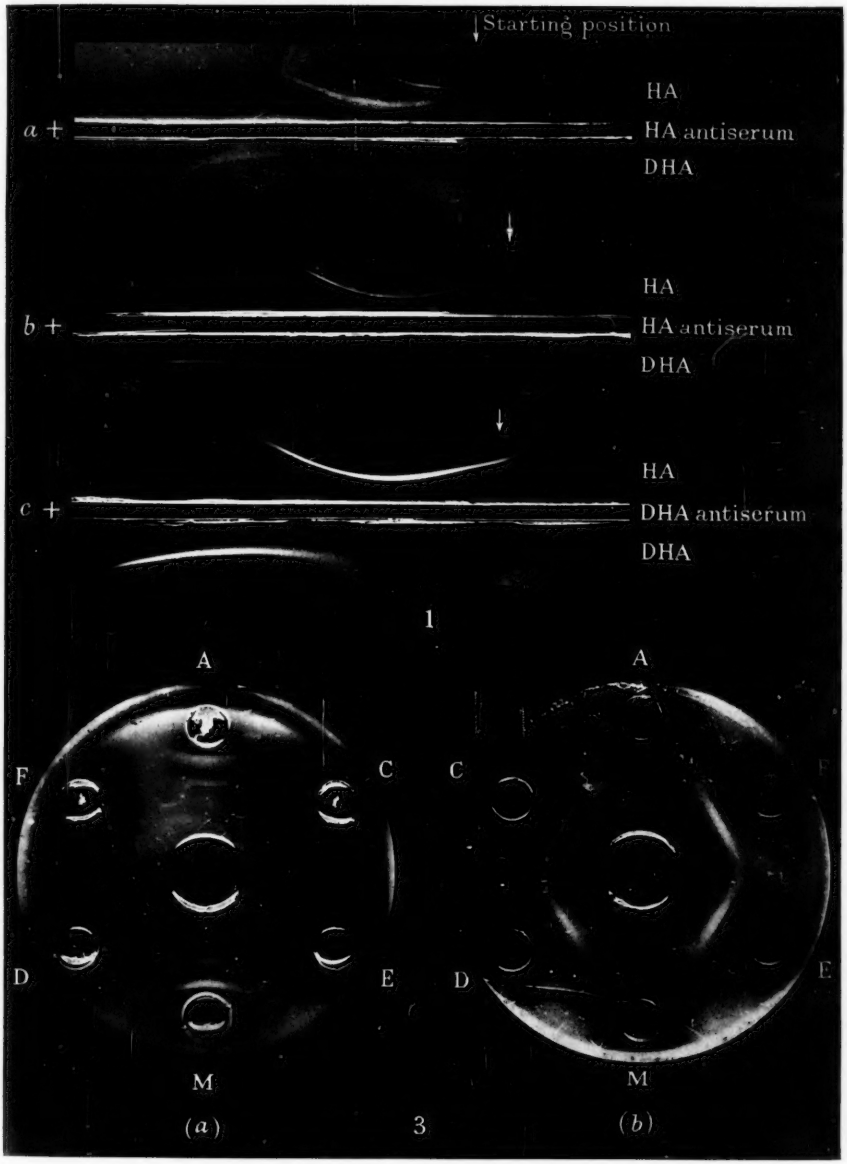
FIG. 2.—Apparatus for electrophoresis in agar. (1) Lid. (2) Electrode compartments for Ag/AgCl electrodes. (3) Buffer vessels. (4) Tray for the agar plate. (5) Connection between electrode compartment and buffer vessel. (6) Ag/AgCl electrode. (7) Electrode retaining nut. (8) Aperture in the lid for the electrode. (9) Slot locating the electrode.

Electrophoresis in agar (Grabar and Williams, 1953).—The apparatus used is shown in Fig. 2. Reversible Ag/AgCl electrodes surrounded by 0·75 per cent agar in water saturated with KCl were employed. Electrophoresis was carried out in 3 per cent agar in glycine buffer (pH 8·4, I = 0·05). Solutions subjected to electrophoresis were made up in glycine buffer.

EXPLANATION OF PLATE.

FIG. 1.—Electrophoresis in agar of H.A. and D.H.A. (glycine buffer pH 8·4 I = 0·05) followed by diffusion from a longitudinal channel of H.A. antiserum (*a*, *b*) and D.H.A. antiserum (*c*).

FIG. 3.—The demonstration of antigen (H.A.) and antibody by diffusion in agar. Peripheral cups contain sera from rabbits of litter 695 (106th day after birth, 5th day after last injection). Rabbits C, D, E, F were injected at birth with H.A. (*a*) Centre cup contains H.A. (*b*) Centre cup contains H.A. antiserum.



Cinader and Dubert.

Sulphur analysis.—Protein, dried *in vacuo* at 105° to constant weight, was analysed for sulphur by Messrs. Weiler and Strauss.

Examination of Cross-reacting Antigen-antibody Systems.

The composition of an antiserum containing antibodies to different groups can be examined by inhibition of agglutination.

Tanned sheep erythrocytes were sensitised with the complex antigen of multiple specificity and were agglutinated with an antiserum to the complex antigen. The agglutination titre so obtained is reduced if cross-reacting antigens are added to the immune serum.

The complex antigen used in our experiments was D.H.A. and the inhibiting antigens were H.A. and D.R.A. D.H.A. antisera heated at 56° and absorbed with sheep erythrocytes were mixed with H.A. or D.R.A. and the mixture was diluted with polyvidone saline to a final serum dilution of 1/10. The mixtures were kept for 2 hr. at 37° followed by 24 hr. at 2° and were then centrifuged. The agglutination titre of the mixture was determined and compared with that of the immune serum which had not been incubated with antigen.

RESULTS.

The Response to Injections with Human Albumin (H.A.).

Three litters of rabbits were used; two (693, 695) were the results of chance matings and only in the third (712) was the father known. Of 16 new-born rabbits, 10 were immediately injected with human albumin (H.A.) and 6 (2 in each litter) were kept as controls (Table I). All rabbits are identified by the number of the litter and a letter. In each litter A and B were controls and were not injected during the first weeks of life. The three mothers (693M, 695M, 712M) and the father (712P) received the same injections as the controls throughout the experiment with H.A.

TABLE I.—*Injections of Human Albumin given to New-born Rabbits.*

Injected intraperitoneally (i.p.) in 0·15 M-NaCl and subcutaneously (s.c.) in emulsion. Amounts injected in mg.

Litter.	Rabbit.	Time, route and amount of injections. First injections.	Subsequent injections.
693	A, B	—	—
	C, D, E	Within 24 hr. 100 i.p. Ditto 400 s.c.	2nd day 260 s.c.
695	A, B	—	—
	C, D	Within 24 hr. 300 i.p.	1st day 120 i.p. 2nd day 90 i.p. 3rd–9th day each 100 i.p.
	E, F	Ditto 300 i.p. „ 270 s.c.	3rd day 300 i.p. 12th day 200 i.p.
712	A, B	—	—
	C, D, E	After 4½ hr. 1·15 s.c.	1st–19th day each 1·15 s.c.

During the first three weeks of life the new-born rabbits were weighed daily. Growth rates from the 1st to the 13th day were computed by plotting weight against age and by fitting straight lines by the method of least squares. The slopes obtained were compared by *t*-tests. There was no significant difference in the growth rate of the injected and the untreated animals of litters 693 and

695 (693 : $t = 1{\cdot}52$, $\cdot 3 > P > \cdot 2$; 695 : $t = 0{\cdot}665$, $\cdot 5 > P > \cdot 6$). The injected rabbits of litter 712 appeared to have a significantly higher growth-rate ($t = 4{\cdot}19$; $\cdot 02 > P > \cdot 05$) than the control animals, probably due to the relatively large volume of liquid injected (15 ml. in 21 days) and slow elimination.

In all the Tables sera are identified by the day of bleeding counted from birth. The sera from other rabbits are similarly designated, the days being counted from the birth of the litter with which they are included.

About two months after birth all animals were bled ; sera from rabbits of litter 693 (58th day) were tested for the presence of H.A. and none could be detected (Table II).

TABLE II.—*Inhibition of Agglutination of Tanned Erythrocytes Sensitised with H.A. by H.A. in Rabbit Sera.*

	Solution added to standard antiserum.	Day of Last injection.	Day of Bleeding.	Titre.
	Normal rabbit serum	—	—	32,000
	Polyvidone saline	—	—	32,000
Normal rabbit serum containing μg. H.A./ml.	1	—	—	32,000
	2	—	—	16,000
	20	—	—	8,000
	40	—	—	8,000
	80	—	—	4,000
Sera from rabbits injected with H.A. at birth	693C	2	58	32,000
	693D	2	58	32,000
	693E	2	58	32,000
	712C	117	122	4,000
	693C	122	128	4,000

After the first bleeding all the rabbits received two to three courses of intravenous injections with H.A. (Table IIIa) ; all sera were examined by agglutination (Table IIIb) and after the last course by precipitin tests (Table IIIc). None of the rabbits injected with H.A. immediately after birth developed a measurable quantity of antibody up to the 261st day after birth in response to later injections with the same antigen. The presence of H.A. in all their sera could be demonstrated by diffusion in agar against H.A. antiserum (Fig. 3) ; in two of them it was determined by inhibition of agglutination (Table II). The serum (693C, 128) of one of these two rabbits (weight 2700 g. and injected with 40 mg. of H.A. between the 118th and 122nd days) contained 0·08 mg. H.A./ml. on the 128th day, that of another animal (712C, 122) (weight 2600 g. and injected with 30 mg. of H.A. between the 113th and 117th days) contained 0·08 mg. H.A./ml. serum on the 122nd day (Table II). Of the controls, one rabbit 695B died before antibody could be detected and another 712A did not form antibody after several courses of injections. Unlike sera from rabbits injected at birth and that of rabbit 712A, the serum of 695B did not contain H.A. 9 days after the first course of injections.

Diffusion in agar of the majority of H.A. antisera against excess H.A. gave more than one line, but sera from rabbits injected at birth with H.A. gave no lines. The antibody response to the minor as well as the major components of the albumin preparation was, therefore, affected.

TABLE III.—*Antibody Response to the Injection of H.A.*

Rabbits C, D, E, F injected with H.A. at birth, rabbits P, M, A, B not injected at birth.

(a) Dose.

Litter.	693.			695.		712.		
Injection of H.A. days after birth.	69, 70, 71, 72, 73	94, 96, 98	118, 120, 122	72, 74, 76	97, 99, 101	66, 68, 70	88, 90, 92	113, 115, 117
Total mg. H.A.	25	30	40	30	30	30	30	30

(b) Agglutination titre of tanned erythrocytes sensitised with H.A.

Bleeding: days after birth		83	103	128	86	106	80	97	122
Rabbits	P	*	*	*	*	*	800	20,000	50,000
	M	40	160	2,000	1,000	8,000	<2	2,000	20,000
	A	320	10,000	50,000	800	10,000	<2	<2	<2
	B	80	4,000	10,000	<2	Died	200	15,000	50,000
	C	<2	<2	<2	<2	<2	<2	<2	<2
	D	<2	<2	<2	<2	<2	<2	<2	<2
	E	<2	<2	<2	<2	<2	<2	<2	<2
	F	†	†	†	<2	<2	†	†	†

(c) Precipitation of H.A. mg./ml. of serum at α-optimum.

Bleeding: days after birth		—	—	128	—	106	—	—	122
Rabbits	P	—	—	*	—	*	—	—	0·37
	M	—	—	—	—	0·10	—	—	0·35
	A	—	—	0·18	—	0·15	—	—	—
	B	—	—	0·13	—	Died	—	—	0·65

* Not available.
† Only five animals in this litter.

The Response to Injections with Diazo-human Albumin (D.H.A.).

The results described so far have established that human albumin (H.A.) injected into a rabbit at birth inhibits the formation of antibody in response to later injections with H.A., and that this inhibition extends to the minor as well as the major constituents of the antigen preparation. The antibody-forming mechanism or the process leading to the release of antibody has been modified.

Chemical substitution can alter the properties of a protein so that its species specificity is reduced and a new specificity appears which is due to the introduced groups. It seemed possible that animals whose antibody response to an antigen had been inhibited by injection of the antigen at birth, might still be capable of producing antibodies to derivatives of that antigen. Such antibodies might differ from those produced by the normal animal and a comparison of the properties of the two could help to explain the inhibition of antibody response in animals injected at birth. Diazo-human albumin (D.H.A.) was chosen as a derivative for injection.

The antibody composition of D.H.A. antisera was explored by their interaction with D.H.A., H.A. and diazo-rabbit albumin (D.R.A.). D.H.A. reacted with all

TABLE IV.—*Injections with Diazo-human Albumin (D.H.A.) and Human Albumin (H.A.).*

Days of bleeding are counted from day of birth, except M, P (the parental rabbits), 620 and 662 (normal adult animals) where the days are counted from the day of birth of the litter with which they are included.

Litter.	Animal.	Protein injected.	Injection. Days after birth.	Quantity in mg.	Route.	Day of bleeding.
693	B	D.H.A.	204	10	i.v.	—
	C*		222, 224, 226	15	i.v.	231
	E*					
	A	H.A.	252, 254, 256	15	i.v.	261
	D*					
	M					
695	620†					
	662					
	C*	D.H.A.	117	6	s.c. (emulsion)	—
	E*		133, 135	6	i.v.	140
	M		161, 163, 165	9	i.v.	169
	A	H.A.	191	4·5	i.v.	196
	D*					
	F*					
712	B	D.H.A.	139	10	i.v.	—
	C*		157, 159, 161	15	i.v.	166
	E*		185, 187, 189	15	i.v.	194
	P					
	A	H.A.				
	D*					
	M					

* Rabbits injected with H.A. at birth.

† Rabbit 620 received in addition to the injections indicated, a s.c. injection of 5·7 mg. D.H.A. in an emulsion containing heat-killed tubercle bacilli on the 100th day.

the antibodies, H.A. with protein specific antibodies and D.R.A. with antibodies to the haptene.

Six rabbits injected at birth with H.A., and proved subsequently to be unresponsive to H.A., were injected 4–8 months later with D.H.A. Other rabbits from the same litters treated in the same way since birth were injected with H.A. Rabbits not injected at birth, some from the same litters, were included in each group (Table IV).

Sera from all these animals were examined by agglutination of tanned erythrocytes sensitised with H.A. and with tanned erythrocytes sensitised with D.H.A. (Table V), by diffusion in agar and some of the positive ones by precipitin tests

TABLE V.—*Response of Normal Rabbits and of Rabbits Injected with H.A. at Birth to Diazo-human Albumin (D.H.A.) and Human Albumin (H.A.).*

	Injected with D.H.A.				Injected with H.A.			
			Agglutination titre, erythrocytes sensitised with :				Agglutination titre erythrocytes sensitised with :	
	Rabbit.	Day of bleeding.	D.H.A.	H.A.	Rabbit.	Day of bleeding.	D.H.A.	H.A.
Not injected at birth	693B	231	500	2,000	693A	231	2,000	10,000
		261	500	1,000		261	2,000	10,000
	662*	140	1,000	2,000	693M	231	10	2,000
		169	5,000	2,000		261	10	500
	620*	169	10,000	20,000	—	—	—	—
	695M	140	2,000	5,000	695A	140	500	20,000
		169	5,000	5,000		169	2,000	50,000
		196	1,000	5,000		196	1,000	50,000
	712B	166	5,000	5,000	712A	166	<2	<2
		194	10,000	10,000		194	<2	<2
	712P	166	5,000	5,000	712M	166	5,000	10,000
		194	5,000	10,000		194	2,000	10,000
Injected at birth with H.A.	693C	231	<2	<2	—	—	—	—
		261	<2	<2				
	693E	231	<2	<2	693D	231	<2	<2
		261	500	<2		261	<2	<2
	695C	140	<2	<2	695D	140	<2	<2
		169	<2	<2		169	<2	<2
		196	<2	<2		196	<2	<2
	695E	140	500	500	695F	140	<2	<2
		169	500	1,000		169	<2	<2
		196	100	200		196	<2	<2
	712C	166	<2	<2	712D	166	<2	<2
		194	<2	<2		194	<2	<2
	712E	166	<2	<2	—	—	—	—
		194	<2	<2				

* Rabbit was not injected with H.A. at any time.

(Table VI). Four out of 10 animals injected at birth with H.A. were injected with H.A. and none formed antibody ; six were injected with D.H.A. and two responded by forming antibody.

TABLE VI.—*Response to Injections with H.A. (Rabbit 695A) and D.H.A.*

Precipitation of antigen (mg./ml. serum) at α-optimum.

Litter		695	695	—	695
Rabbit		M	E	662	A
Day of last injection		165	165	165	165
„ „ bleeding		169	169	169	169
mg. antigen/ml. serum	H.A.	0·075	—	0·13	0·40
	D.H.A.	0·090	0·020	0·23	0·16
	D.R.A.	0·030	—	0·03	—

The H.A. agglutination titres (Table V) were higher in relation to the D.H.A. titres than would have been expected from the precipitin tests with H.A. and D.H.A. (Table VI) ; but the ratio of the agglutination titres of sera from every litter against H.A. and D.H.A. was relatively higher for the immunising antigen. Of the two D.H.A. antisera obtained from animals injected at birth with H.A., one (693E) did not agglutinate erythrocytes sensitised with H.A. and the other (695E) did agglutinate them. An antibody to H.A. could not be demonstrated by flocculation in serum 695E, which differed from other H.A. antisera from rabbits immunised with D.H.A. by giving a marked prozone in agglutinations of tanned cells sensitised with H.A.

The Reaction of Antisera to Diazo-human Albumin with Human Albumin and Diazo-rabbit Albumin.

The agglutination of tanned cells sensitised with an antigen can be inhibited by the addition of antigen to the antiserum. This inhibition occurs only if the agglutination is due to antibody and not when it is due to some non-specific interaction (Stavitsky, 1954). Inhibition tests showed that the D.H.A. titres of sera from 693E and 695E were due to an antibody specific for D.H.A. The agglutination by an H.A. antiserum of tanned cells sensitised with D.H.A. can also be inhibited by the addition of H.A. to the H.A. antiserum . Inhibition is complete when the quantity of H.A. added corresponds to that at the equivalence point of the serum. Smaller quantities of H.A. inhibit only partially (695A, Table VII). On the other hand, the agglutination by a D.H.A. antiserum could generally not be completely inhibited by the addition of H.A. to the serum. The agglutination titre decreased with increasing quantities of H.A. until a constant value was reached in the antigen excess zone. The agglutination titre was the same for 5, 10 or 50 times the equivalent quantity of antigen (Table VII, 695M, 662, 695 E).

The relative shift of the agglutination titre (*cf.* 695M, 169, Tables VII and VIII) was unaffected over a tenfold range by the volume in which antigen and antibody were incubated. Nevertheless some inhibition experiments were made with immune sera which had been diluted initially with normal serum so as to give agglutination titres between 1/500 and 1/1000. Sera so diluted were subsequently incubated in polyvidone saline with varying quantities of H.A. The agglutination titres of sera from four animals not injected at birth with H.A. were reduced in the presence of H.A. On the other hand, the titre of the serum from rabbit 695E which had been

TABLE VII.—*Reciprocal Agglutination Titres of D.H.A. Antisera Mixed with H.A.*

Tanned erythrocytes sensitised with D.H.A. 695A (169) immunised with H.A., 695M (169), 662 (169), 695E (169) immunised with D.H.A., last injection on the 165th day after birth.

Immune serum.			μg. H.A./ml. diluted serum (1/10).													
Rabbit.	Days.	Diluted in normal serum.	0·0	0·46	0·6	0·93	1·23	2	4	4·6	6·7	8	9·3	13	40	67	80
695A	169	1 : 5	1000	—	—	—	—	50	20	—	—	<10	—	—	<10	—	<10
695M	169	1 : 10	500	500	—	200	—	—	—	100	—	—	100	—	—	—	—
662	169	1 : 10	1000	—	500	—	200	—	—	—	100	—	—	100	—	—	—
695E	169	1 : 1	500	—	500	—	500	—	—	—	500	—	—	500	—	500	—

injected with H.A. at birth was not reduced by incubation with H.A., nor was it reduced if the antigen-antibody mixtures were kept for 7 days at 2° before dilution and addition of sensitised cells.

In another experiment (Table VIII) agglutination titres of sera were compared in absence of added antigen, in the presence of excess H.A. and in the presence of excess D.R.A. The titres of D.H.A. antisera from rabbits not injected at birth with H.A. were reduced by the addition of H.A. The agglutination titre of the serum from the second animal injected at birth with H.A. and subsequently immunised with D.H.A. (693E—261) was unaffected by incubation with H.A.

TABLE VIII.—*Reciprocal of Agglutination Titres of D.H.A. Antiserum Mixed with H.A. and D.R.A.*

Tanned erythrocytes sensitised with D.H.A.; 1 mg. antigen was added to 1 ml. of serum diluted 1/10.

	D.H.A. antiserum.		Antigen added to serum.		
Rabbit.	Day of last injection.	Day of bleeding.	—	H.A.	D.R.A.
693B	256	261	500	10	200
693E*	256	261	500	500	100
695M	165	169	5,000	1,000	1,000
712B	189	194	10,000	5,000	10,000
712P	189	194	5,000	500	1,000

* Injected at birth with H.A.

D.R.A. reacts with antibodies of benzene-*p*-sulphonic acid specificity; it reduced to some extent the agglutination titre of most sera from rabbits immunised with D.H.A. The reaction of serum 693E (261) with D.R.A. does not differ quantitatively or qualitatively from that of other D.H.A. antisera.

There was therefore a marked difference between the sera of responding rabbits not injected at birth and those injected at birth which had produced antibodies to D.H.A. The D.H.A. titres of the sera of the latter two animals (693E, 695E) were not reduced in the presence of excess H.A. and are of the magni-

TABLE IX.—*Response to Injections with Tobacco Mosaic Virus.*

Precipitin titres: mg. T.M.V./ml. serum at α-optimum.

Rabbits not injected at birth.				Rabbits injected with H.A. at birth.			
	Day.				Day.		
Rabbit.	Injection.	Bleeding.	mg. T.M.V./ml.	Rabbit.	Injection.	Bleeding.	mg. T.M.V./ml.
693A	238	245	0·38	693C	238	245	0·76
693B	238	245	1·20	693D	238	245	0·19
693M	238	245	0·20	693E	238	245	0·02
695A	205	212	0·38	695C	205	212	1·52
695M	205	212	0·06	695D	205	212	0·38
				695E	205	212	0·76
				695F	205	212	0·76
712A	171	178	0·38	712C	171	178	0·01
712B	171	178	0·05	712D	171	178	0·38
712M	171	178	0·025	712E	171	178	0·20
712P	171	178	0·46				

$t = 0{\cdot}781$ $0{\cdot}5 > P > 0{\cdot}4$.

tude of the weakest serum from rabbits not injected at birth. After the addition of H.A., the D.H.A. agglutination titres of the responding rabbits in the two groups are quite similar.

The Response to Injection with Tobacco Mosaic Virus.

Each rabbit from the litters 693, 695 and 712, including the parents, was injected with 1 mg. T.M.V. and was bled seven days later. Preliminary experiments indicated that the equivalence zone could be adequately determined by finding the α-optimum. The precipitin titres (Table IX) of rabbits injected with H.A. at birth and rabbits not injected did not differ significantly ($0{\cdot}5 > P > 0{\cdot}4$).

DISCUSSION.

Rabbits injected with human albumin shortly after birth did not form antibodies in response to later injections with the same antigen. The human albumin preparation contained trace quantities of two other antigens, and whereas the ten control animals, with three exceptions, formed antibodies against one or both of these antigenic impurities, none of the rabbits injected at birth formed any antibodies demonstrable by diffusion in agar. It follows that quantities of protein lower than 23 mg. of human albumin, the lowest quantity injected into new-born rabbits, may be sufficient to inhibit the antibody response.

The inhibition of antibody response to H.A. persists for at least eight months after birth. It does not affect the antibody response to a completely unrelated protein, since the antibody response to tobacco mosaic virus of the rabbits injected at birth with H.A. and of those not injected was similar. The two groups differed, however, in their response to diazo-human albumin. All the control rabbits were able to form D.H.A. antibodies, but only two of the six rabbits injected with H.A. at birth formed antibody to D.H.A. when injected with D.H.A. The serum from one of these two rabbits (695E) agglutinated tanned sheep erythrocytes sensitised with H.A. This appears to be a breakdown of the inhibition of immune response to H.A.

It is possible to consider antibody to D.H.A. as consisting of antibody to H.A. and antibody to the benzene-*p*-sulphonic acid group; both these antibodies can react with D.H.A.

Addition of H.A. to D.H.A. antisera reduced the anti-D.H.A. titre of sera from all the controls, leaving a residual titre in all but one of them; even this one D.H.A. antiserum, which was completely inhibited by H.A., was partly inhibited by D.R.A. and therefore contained some antibodies to the benzene-*p*-sulphonic acid group. In contrast, the D.H.A. antisera from the two rabbits injected at birth were not inhibited by H.A.

This contradictory finding may be due to a very low avidity of the H.A. antibody, allowing the complex between the antibody and the antigen in solution to dissociate readily and enabling the antibody to react instead with the antigen on the erythrocytes. It has been suggested (Haurowitz and Schwerin, 1943) that antiserum to a substituted antigen contains not only antibody to protein and the substituent groups, but also antibody specific for the substituent group itself, including its site of attachment to the protein. Antisera to D.H.A. from animals injected at birth with H.A. may contain only the latter two types of antibody. It is possible that an antibody to the substituent group, including its site of attach-

ment on the protein, would behave as a non-avid antibody when reacting with the unaltered protein.

The inhibition of antibody response of the animal could be due to the presence of the antigen in the antibody-forming cells. The antigen could have entered the immature cells and consequently be located in positions within the matured cell which are not normally accessible to antigen. The antigen present in these cells might retain antibody within the cell. It would thus only affect the release of antibody which is capable of combining with the antigen present in the cell. On this basis, D.H.A. would not give rise to H.A. antiserum, but would give rise to a normal level of antibody to the benzene-*p*-sulphonic acid group. The results do not support this suggestion, since four out of six animals injected at birth with H.A. were unable to form D.H.A. antibodies.

An animal injected with an antigen at birth may in later life exclude that antigen from the sites of antibody formation, or it may be unable to synthesise antibody to the antigen even after the antigen has reached the sites. A mechanism for the second of these possibilities has been proposed by Burnet and Fenner (1949). Either possibility poses the problem of the recognition and identification of the antigen by or within the sites of antibody formation. Antigens might be identified by the antibody-forming mechanism as homologous by the overall size or shape of the molecule as a whole, or only the identification of limited areas on the surface of the molecule. In the former case, it would be possible for a rabbit to produce an H.A. antiserum when successfully immunised with a derivative of H.A. In the latter, this would be impossible except in the form of a cross-reacting antibody. Though our evidence on this point is not decisive, the formation of antibodies to the haptene in rabbits injected with H.A. at birth and immunised with D.H.A. has been definitely established, and tends to support the view that the antigen is recognised by individual surface areas on the antigen. There is every indication that further experiments with modified proteins may add to the insight into the processes involved in antibody formation.

SUMMARY.

New-born rabbits from three litters were injected with human albumin which contained traces of two alpha globulins. None of the rabbits so injected responded to human albumin injections during adult life with the formation of antibody either to the main component or to the trace components. Of the controls all but one produced antibody.

All the rabbits were finally injected with tobacco mosaic virus, and produced antibody. There was no significant difference between the response of the animals injected at birth with human albumin and those not so injected.

Six of the rabbits injected at birth with human albumin were injected with benzene-*p*-sulphonic acid-azo human albumin and two of them produced antibodies to this antigen. The nature of these antibodies has been investigated and it has been shown that they are essentially directed against the benzene-*p*-sulphonic acid group; they have little or no avidity for human albumin.

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THE EFFECT OF X-RAYS ON THE SECONDARY ANTIBODY RESPONSE.

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MANY investigators have shown that a sublethal dose of X-rays interferes with a primary antibody response if the antigen is given after the X-rays; the subject has been reviewed by Taliaferro and Taliaferro (1951). Rather fewer investigations have been made on the effect of X-rays on the secondary response and opinion on this matter seems divided (Taliaferro and Taliaferro, 1954; Silverman and Chin, 1953; Dixon, Talmage and Maurer, 1952).

The Army Pathology Advisory Committee recommended that wounded soldiers who have been actively immunised should be given a dose of tetanus toxoid and not tetanus antitoxin as had been the practice previously, and this recommendation was accepted (Sachs, 1952). The question later arose whether, after exposure to X-rays, re-immunisation was still the best treatment for those at risk from tetanus.

With these facts in mind, the present work describes two experiments on rabbits given X-rays at varied times and doses before a second dose of tetanus toxoid.

MATERIALS AND METHODS.

Each of the two experiments was performed on comparable groups of 3 rabbits inoculated intramuscularly with the same batch of formol tetanus toxoid, strength 8 Lf/ml., on day 0 and day 45. Irradiation when given was from a 240 kV Westinghouse constant potential machine, the distance between target and entry surface being 1 m. The area of uniform dosage was 35 × 28 cm. using a ½ value layer of 1·2 mm. of copper. Bilateral irradiation to the whole body at a rate of 23 R/min. was used throughout as suggested by Chapman and Barnes (1953).

In the first experiment 400 R were given either 10 days, 2 days or 6 hr. before the second dose of toxoid and in the second experiment either 100 R or 25 R were given 10 days before the second dose of toxoid. One group in each experiment was not irradiated and served as a control.

The serum antitoxin was titrated at 10–20 per cent intervals using the mouse method described by Glenny and Stevens (1938). Bleedings for this purpose were taken on day 45 immediately before the second dose of toxoid and subsequently as follows:

	Day of bleeding for antitoxin titre.											
Exp. I . .	47	—	49	51	53	55	58	61	65	70	90	120
Exp. II . .	47	48	49	51	53	56	—	60	65	72	—	—

The secondary response for each group was judged by the following criteria:

1. The time of maximum rate of rise/day in antitoxin titre.
2. The time of maximum serum antitoxin concentration (peak titre).
3. The maximum overall logarithmic rise in titre from day 45.

As the number of animals used is small, the just significant difference ($P = 0{\cdot}05$) is calculated from the pooled variations within the groups for both experiments combined.

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RESULTS.

The effect of a second injection of tetanus toxoid 45 days after the first injection and its modification by X-rays is shown in Tables I and II and will be considered under three headings.

TABLE I.—*Exp. I: Effect on Antitoxin Production of* 400 R *given at Various Intervals before Day* 45 *(Second Dose of Toxoid).*

Interval between X-rays and toxoid.	Time in days for: Max. rate antitoxin rise/day.	Mean.	Max. titre.	Mean.	Overall rise in titre (log).	Mean
10 days	9	8·3	20	16·7	0·41	0·32
" "	7		20		0·26	
" "	9		10		0·30	
2 "	5	6·3	16	27	0·97	1·09
2 "	9		45		0·58	
2 "	5		20		1·71	
6 hours	9	5·7	16	16	1·28	1·45
6 "	3		16		2·12	
6 "	5		16		0·95	
Controls	3	3·0	6	6·7	1·51	1·54
"	3		8		1·51	
"	3		6		1·61	
Just significant difference $P = 0·05$		2·7 days		5·8 days		0·92

TABLE II.—*Exp. II: Effect on Antitoxin Production of Various Doses of X-rays given* 10 *Days before Second Dose of Toxoid.*

Dose of X-rays.	Time in days for: Max. rate antitoxin rise/day.	Mean.	Max. titre.	Mean.	Overall rise in titre (log).	Mean.
100 R	5	4·0	11	12·3	1·07	1·09
" "	3·5		15		1·05	
" "	3·5		11		1·16	
25 R	3·5	3·5	11	9·7	0·77	1·02
" "	3·5		6		1·18	
" "	3·5		11		1·12	
Controls	2·5	2·5	8	10·3	2·90	1·62
"	2·5		8		0·84	
"	2·5		15		1·08	
Just significant difference $P = 0·05$		2·7 days		5·8 days		0·92

1. *The time of maximum rate of rise in antitoxin/day.*

In all the control rabbits there was a period of 2 days before the antitoxin titre started to rise significantly and the rise, when it occurred, was at first rapid and then lessened. The maximum rise per day in the controls took place between the 2nd and 3rd day in experiment II, but in the first experiment, as the titre was only done on day 49, the maximum rise could not be timed with such certainty.

In all the irradiated animals the maximum rise was postponed. The overall difference is highly significant ($P < 0{\cdot}01$) and it will be seen that as the interval between X-rays and toxoid increased up to 10 days so also the average time before the maximum antitoxin rise per day increased.

2. *Time of peak titre.*

The interval before peak titre in both experiments was variable, 5 out of 6 control rabbits achieving peak titre within 8 days. In all the rabbits irradiated with 400 R this interval was prolonged ($P < 0{\cdot}05$). With smaller doses of X-rays the delay in reaching peak titre was less significant.

3. *The total secondary rise in antitoxin titre.*

The overall rise varied considerably within different experimental groups. Only when 400 R were given 10 days before the second dose of toxoid was the peak titre depressed significantly in all animals.

DISCUSSION.

As in the primary response the action of X-rays is twofold. There is the delaying action on the commencement of antitoxin rise and peak titre, and there is reduction in the ability to form antitoxin. The former may occur without the latter and these effects will be called " delay " and " reduction ".

Dixon *et al.* (1952), observing the antigen disappearance rate of ^{131}I-labelled bovine gamma globulin as well as precipitin formation in response to it, found delay only in the secondary response when the antigen was given two days after 400 R. Taliaferro and Taliaferro (1954), studying the formation of sheep cell haemolysin in rabbits, found delay and reduction in the secondary response when the antigen was given 2 days after 500 R but delay only when given 1–2 hours before. Silverman and Chin (1953), measuring egg albumen precipitin, found no reduction in peak titres in the secondary response when the antigen was given 24 hours after 400 R. Their figures suggest, however, that there was some delay. In the present experiment there was delay when the antigen was given 6 hours, 2 days or 10 days after 400 R, reduction in all rabbits when the interval was 10 days, and probably reduction in one of the three rabbits at the 2 days' interval.

The inference from these results is that the effect of X-rays in causing diminution of peak titre takes two days or more to develop. If, for example, it took only one day, then Silverman and Chin would have found reduced peak titres.

Taliaferro and Taliaferro (1954) showed in the primary response that the ability to form antibody is lost very rapidly when the antigen is given at the time of irradiation, or up to one day later. In the secondary response this sharp break in antibody-forming ability, if it occurs, is probably two days later, *i.e.*, when the antigen is given 2 days after the X-rays. The sharpness of the break explains the different results of Dixon *et al.* and Taliaferro. The start of the antibody rise is nearly 2 days earlier in the secondary response studied by the Taliaferros and perhaps this is the radio-sensitive period. If so, then the " reduction " damage by X-rays takes 9 days to develop in this experiment ; unless the time of antibody rise is 9 days or more after 400 R no reduction in peak titre occurs. The same applies to the Taliaferros' work on both primary and secondary response.

The delay effect occurs more readily ; persons exposed to radiation may be

expected to show a delay in antitoxin rise if given a booster dose of tetanus toxoid. The value of giving a stimulating dose of toxoid to those at risk from tetanus depends on a rapid rise in antitoxin titre within the incubation period of the disease. The findings in these experiments suggest that this procedure may be of doubtful value in irradiated persons.

SUMMARY AND CONCLUSIONS.

The action of X-rays on the secondary response in rabbits to tetanus toxoid has been studied. This response is radio-sensitive but differs from the radio-sensitivity of the primary response in that a dose of 400 R has to be given two or more days before the antigen to reduce the peak titre. There is delay in antitoxin rise whether the X-rays are given 6 hours, 2 days or 10 days before the antigen.

Smaller doses of X-rays (100 and 25 R) given 10 days before the antigen may delay the antitoxin response but have very little effect on the peak titre.

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THE RATE OF ADSORPTION OF BACTERIOPHAGE BY ROUGH AND SMOOTH STRAINS OF *SALMONELLA TYPHI*.

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IN a previous paper (Fildes, 1954) it was shown that lysis by phage of susceptible smooth cultures of *Salm. typhi* was absent or little marked under the same conditions as allowed rapid lysis of rough cultures. It was pointed out that visible lysis cannot take place until the numbers of bacteria and phages are more or less equal and that this cannot occur from a small initial concentration of phage during the log phase of growth if, for any reason, the rate of phage production is slow. It appeared to be slower with smooth cultures than with rough. In the meantime it was noted that the titre of a single specimen of phage varied markedly according to the " lawn " on which it was plated. Using the 2-layer method the number of phages per ml. was 7·2, 7·0, 6·1, 5·6, 5·2 and $5{\cdot}0 \times 10^9$ on six different rough strains, and 3·5, 3·2 and $2{\cdot}9 \times 10^9$ on three smooth.

The production of a plaque on a sensitive lawn demands that phage shall be produced at a certain rate. It is a matter of a series of phage production cycles sufficient to lyse a visible area, taking place before the surrounding bacteria have ceased growth. If for any reason the overall production-rate of phage on one lawn is greater than on another, more plaques will reach a visible size on the former than on the other.

The evidence of plaque counts thus confirmed the conclusion from the lysis experiments that in smooth strains overall phage production-rate is less than in rough.

The present paper shows that the cause of the overall slowness with smooth bacilli is slowness of adsorption.

MATERIALS.

Phage.

It was necessary to select a phage for study which was active in spot tests against representative rough and smooth bacteria. Two were available, Felix's international standard " anti-O " phage which appears to be identical to our phage designated " Phage 2 ", and our " Phage 1 ". The difference in gross lysis between rough and smooth strains is readily shown with Phage 2 (Fildes, 1954), but this phage is less satisfactory for adsorption experiments because the bacterial receptors are less stable than those of Phage 1 (Kay, 1955). For this reason we have used only Phage 1 in the following experiments. This phage was referred to by Fildes (1954) and Kay (1955). It was found associated with " Phage 2 " in an " anti-O " phage preparation active against No. 3390 in the National Collection of Type Cultures. Phage 1 and Phage 2 are active against most typhoid bacilli, rough, smooth or Vi, but Phage 2 is totally inactive against " Bact. 1 " the type strain of Phage 1, which was derived from N.C.T.C. No. 3390 also.

Striking differences between the two phages are that Phage 1 remains active against all Phage 2-resistant mutants, while Phage 1 is inactive, but Phage 2 active, against " P ", another derivative from N.C.T.C. 3390, Felix's ViI.R and the Vi strain D1. The preparation of Phage 1 used had been concentrated and purified by washing with low and high speed centrifugation.

Bacteria.

The terms " rough " and " smooth " are used in this paper in their original and physical sense supported by the effects of anti-rough phages T3 and anti-R. As is well known, " on occasion, serologically rough forms may look smooth, and morphologically rough forms may still contain smooth antigens " (Kauffmann, 1954). In the present case, however, the bacteria tested were for the most part classical examples of their types.

Smooth strains.

El (Vi) : This was the well known strain Ty2 (*cf.* Craigie and Felix, 1947).

2256*a* (non-Vi) : This was isolated by Felix from a case of laboratory infection due to a non-Vi strain.

O 901 : Felix's standard non-motile " O " strain.

Rough strains.

Ty2.R, a : This strain sometimes produced low titre anti-O serum (Felix, personal communication).

Mrs. S. R : This had never shown traces of O antigen (Felix).

6 S.R : (Felix).

O 901.R : Selected by us from O 901.S.

R4 : Selected by us from N.C.T.C. No. 3390.

Bact. 1 : Also selected from N.C.T.C. No. 3390. The type strain for Phage 1.

Adsorption Experiments.

The measurement of adsorption of phages by bacteria is based on the counting of free phages at intervals in a phage-bacterium mixture and comparing the result with the initial input of phage. Where adsorption is slow the experiment must continue for a long time during which it must not be vitiated by even small bursts. A simple way to prevent bursts is to stop metabolism by using bacterial suspensions washed in buffer containing the electrolytes necessary for normal adsorption.

The bacteria were grown in a tryptic digest meat broth containing 0·002 M–Mg, 0·00025 M-Ca, and 1/10,000 albumin (Blood Products Laboratory, Lister Institute, London). At a Spekker reading 0·600 the growth was centrifuged and the deposit washed twice in 0·033 M phosphate buffer containing the above concentrations of Mg, Ca and albumin. The deposit was made up with the buffer to half the original volume, approximating to 8×10^8 bacteria/ml. These suspensions were not stable in all cases for very long, the rate of adsorption becoming progressively slower. The results recorded are taken from 24-hr. preparations.

The suspension of Phage 1 for the tests was diluted in the same buffer to 2×10^4/ml. This remained stable for a very long time. The technique of all these experiments followed the same pattern :

Tube 1 contained 4 ml. buffer + 4 ml. phage
„ 2 „ 3 „ „ + 4 „ „ + 1 ml. bacterial suspension.

At time 0, 2 and sometimes 3 samples were taken from tube 1, diluted 1/10 with buffer and kept on ice. At the end of a short experiment, or at intervals during a long, further samples were taken. From tube 2, samples were taken at suitable times, diluted 1/10, centrifuged and the supernatant kept on ice.

At the end of the experiment all samples were plated by the two layer method on a lawn of Bact. 1 which had been freshly grown on the meat broth mentioned above. Next day the plaques were counted, and since the system used required removal of the lid and viewing from the bottom the plates were put into 2 per cent formalin for 10 minutes.

The plate counts from tube 2 gave the number of phages still free at certain times. The initial count was determined from tube 1 and subsequent counts expressed as percentages

of this. The $\log_{10}$ of the percentage free at various times ideally fell on a straight line over the range 0–90 per cent adsorption. The rate of phage inactivation by bacterial extracts was determined in the same way but the centrifugation was omitted.

RESULTS.

Rates of Adsorption.

Fig. 1 shows the rates of adsorption of all the bacteria, as the straight lines approximating to the observed points, which are omitted. The Figure is compiled

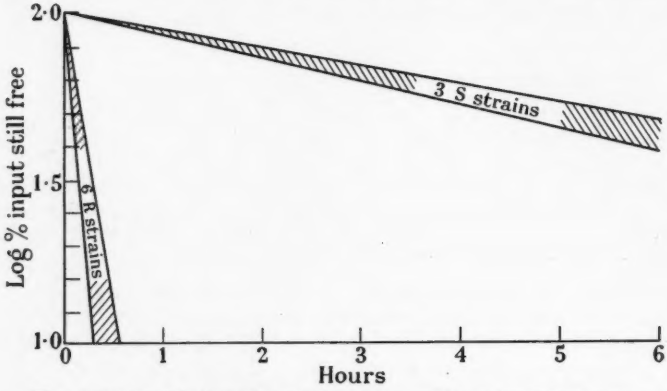

FIG. 1.—Rate of adsorption of Phage 1 by rough and smooth strains.

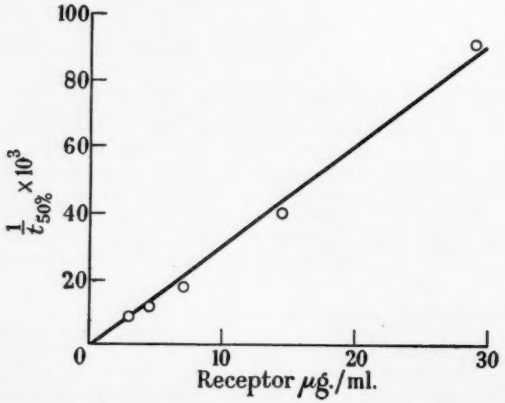

FIG. 2.—Relation of phage receptor concentration to rate of adsorption of Phage 1.

from 5 different experiments. It will be seen that all rough bacteria adsorbed Phage 1 at approximately the same rapid rate, while the smooth were much slower. The rate of adsorption of a phage depends on collisions between the phages and receptors on the bacteria. If these latter are few in number, the number of collisions in

unit time will be less and therefore the rate of adsorption slow. This conclusion was checked by using a relatively pure preparation of isolated Phage 1 receptors (Kay, 1955). The 50 per cent adsorption time was determined with 5 concentrations of phage receptor and the reciprocals of the times found ($\times 10^3$) were plotted (Fig. 2). It is seen that, taking into account unavoidable experimental error, 50 per cent adsorption is attained in times inversely proportional to the receptor concentration.

On these grounds the rate of adsorption may be taken to be a measure of the number of receptors on a bacterium which are available to a phage.

Since O 901.R adsorbed 50 per cent of offered phage in 6 minutes and O 901.S in $4\frac{1}{4}$ hours, it follows that O 901.R has 42 times more receptors available than O 901.S.

CONCLUSION.

We conclude from this work that a rough organism being susceptible not only to Phages 1 and 2 but also to phages anti-R and T3 has not only a greater variety of phage receptors on its surface than a smooth but also a greater number of receptors for Phage 1 which is common to both. The effect of this is to make a rough organism much more susceptible to lysis since collisions are more frequent between phage particles and receptors.

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SURVEY OF PAPERS

HOWARD has studied the neurotoxin of *Sh. shigae*. He finds that it kills mice more readily when given intravenously rather than intramuscularly or intraperitoneally, and that rabbits die more rapidly after intravenous inoculation than after injection into the medulla oblongata. Direct injection into the sciatic nerve is without effect. The conclusion suggested from this and other evidence is that the toxin acts by causing damage to blood vessels in the central nervous system, neuronal destruction being secondary to this (p. 439).

BRIDGWATER, MORGAN, ROWSON and PAYLING WRIGHT describe in detail the histological changes in the central nervous system resulting from the intravenous injection of Shiga neurotoxin in rabbits. These mainly affected the cervical and lumbar enlargements of the cord, and the grey more than the white matter, and were primarily vascular, neuronal changes appearing later. These lesions were produced unilaterally when the injection was made into a vertebral artery. Injection direct into the medulla had only local effects and into nerve trunks almost none. It was shown that the toxin has no effect on peripheral neuromuscular conduction. Shiga toxin appears therefore to act on blood vessels in the nervous system and is to be regarded not as a primary but as a secondary neurotoxin (p. 447).

HARRIS describes a study by quantitative methods of certain requirements for growth *in vitro* of rat connective tissue cells. Stored serum in which a precipitate has formed supports no growth: its capacity to do so was restored by adding L-cystine or L-cysteine, but not by any of 17 other amino acids. The two effective substances enhanced growth in fresh serum and enabled growth to develop from a smaller initial number of cells. Less growth-promoting effect was given by D-cystine and D-cysteine: of other sulphur-containing substances tested, thioglycollic acid and sodium sulphite promoted growth, while methionine and several other amino acids did not (p. 454).

SMITH, KEPPIE and STANLEY describe an investigation which they claim has led to the demonstration of a specific lethal toxin produced by *B. anthracis*. Some of the properties of this toxin, which is found in the plasma of guinea-pigs dying from anthrax, are described, and a relationship is postulated between the toxin and the non-toxic protective antigens of the anthrax bacillus (p. 460).

WIDDICOMBE, HUGHES and MAY have examined the efficiency with which the popliteal lymph node of the rabbit removes *B. anthracis*, *B. subtilis* and rabbit erythrocytes from the afferent lymph. Among factors involved, they find that removal of organisms is related to the rate of lymph flow (p. 473).

WATSON and FLOREY performed gastrectomy in rats and subsequently added to the food minute but appropriate amounts of vitamin B_{12} containing ^{60}Co so that the amount absorbed could be determined by estimating the radioactivity of the faeces. Whereas normal animals excreted 66·5 per cent, gastrectomised excreted 93·8 per cent, and the addition of an extract of rat stomach reduced excretion by the latter to 69·5 per cent: on the other hand, pyloric juice from the pig had no consistent similar effect, possibly because intrinsic factor is ineffective in another species. Some unexplained changes in the mucosa of the stomach remnants are also described (p. 479).

JOAN R. DAVIES and WRIGHT have studied the immediate protective action of tetanus toxoid against the lethal effect of tetanus toxin in mice, the variable investigated being the dose of each substance and the interval elapsing between the injections. Protection was only obtained when toxoid was given simultaneously with or 12 hours before toxin, and the dose of the former had relatively to be very large. The findings are held to suggest that toxoid competes with toxin for some receptor substance. Mice incompletely protected and dying late, instead of exhibiting spasm and hyperexcitability, were paralysed (p. 487).

WOODRUFF and SIMPSON have shown that rats injected with homologous cells on the day of birth are, when they grow up, almost completely tolerant of skin grafts from the same donor. Injection at 2 weeks of age is effective in a few recipients, while injection at 4 weeks old induces a state of immunity. These results are not materially affected by large doses of cortisone (p. 494).

DAVIES, SHILO and HESTRIN have shown that intravenous injection of levan in rabbits prevents the subsequent diffusion of trypan blue into sites of skin injury. In guinea-pigs levan prevented the neutralisation by diphtheria antitoxin of toxin injected intradermally. It is suggested that levan acted in these experiments by diminishing capillary permeability (p. 500).

SWAIN describes a morphological study of the multiplication of psittacosis virus, carried out by phase contrast microscopy of suspensions of infected mouse spleen and by electron photomicrography of material from chick embryo yolk sacs. The findings support the view that reproduction is by binary fission (p. 507).

CINADER and DUBERT have studied the specificity of the immune tolerance resulting from inoculation with an antigen during foetal or early post-natal life. New-born rabbits given injections of human albumin failed to produce antibody to this protein when re-injected with it up to 8 months later. They responded normally to injection of tobacco mosaic virus. Of 6 rabbits possessing immune tolerance to human albumin only 2 subsequently reacted by antibody formation to injections of benzene-*p*-sulphonic acid-azo human albumin : evidence is presented that this antibody reacted with the haptene rather than with the albumin itself (p. 515).

CROSLAND-TAYLOR gave rabbits 2 injections of tetanus toxoid 45 days apart, administered a 400 R dose of X-rays at various intervals before the second dose, and assayed the antitoxin in the blood at intervals after it. This dose of X-rays not only delayed the secondary response but, if given 2 or more days before the toxoid, reduced the peak titre. Smaller doses only delayed the response (p. 530).

FILDES and KAY have sought an explanation for the fact that rough cultures of *Salm. typhi* are much more susceptible to lysis by phage than smooth. By performing serial phage counts on mixtures of phage and smooth and rough strains they have shown that adsorption by smooth strains is much slower. Reasons are adduced for concluding that this is due to the presence of a smaller number of receptors on the surface of the smooth bacterial cell (p. 534).

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